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Adult human glial cells can present target antigens to HLA-restricted cytotoxic T-cells

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Summary

T-lymphocyte recognition of antigen either on antigen-presenting cells (APC) necessary for the generation of an immune response or on target cells during the effector phase of a cellular immune response requires expression of HLA molecules. Although immune mechanisms operate in many disease processes of the central nervous system (CNS), cells of the CNS generally express low levels of HLA molecules. In this study, the potential for upregulation of HLA molecules on adult human glial cells was examined. Moreover, the functional implication of this upregulation was assessed by the capacity of glial cells to process and present target antigens to HLA class I-restricted influenza-specific and class II-restricted myelin basic protein (MBP)-specific CTL lines. Glial cells cultured from adult human surgical brain specimens or cells from established glioblastoma multiforme cell lines were studied. Lysis by antigen-specific CTLs was dependent on treatment of the target cell with interferon-γ. The lysis was HLA restricted and antigen specific. The results indicate that adult human glial cells can process and present antigen to HLA-restricted CTLs but require the upregulation of HLA molecules. These findings have implications for infectious and autoimmune diseases of the CNS.

Introduction

Antigen-specific T-lymphocytes are believed to be involved in the production of immunopathological disease of the central nervous system (CNS) such as multiple sclerosis (MS) (Mokhtarian et al., 1984; Fontana et al., 1987). Direct damage of tissue by cytotoxic T-lymphocytes (CTLs) requires recognition of a processed antigen on the target cell in the context of the appropriate HLA determinants (Zinkernagel and Doherty, 1974; Sun et al., 1988). This process may be limited in the CNS, since brain cells express very low levels of HLA molecules (Williams et al., 1980). Expression

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of these molecules can be upregulated in murine glial cell cultures by interferon-γ (IFN-γ) (Wong et al., 1984) or virus infection (Massa et al., 1986) which allows these cells to function as antigen-presenting cells (APC). Animal experiments have demonstrated that mouse endothelial cells (McCarron et al., 1985) and rat astrocytes (Fontana et al., 1984) can present myelin basic protein (MBP) to encephalitogenic T-cell lines. These lines have been shown to lyse MBP-treated rat astrocytes in an antigen-specific and Ia-restricted fashion (Sun and Wekerle, 1986). In addition, inducibility of Ia on rat astrocytes has been found to be strain dependent (Massa et al., 1987). While these findings indicate that native cells of the newborn murine CNS can present self antigen to T-cells, it is uncertain if similar processes occur in cells of the adult human brain and whether these cells can process and present viral antigen to CTLs. Moreover, there is evidence that certain cells such as keratinocytes and epithelial cells can be induced to express Ia molecules but are incapable of processing and presenting antigen to T-cells (Geppert and Lipsky, 1985). In this study, the capacity of adult human glial cells to express HLA molecules and to acquire antigen-presenting function was examined by their ability to serve as targets for HLA class I- and class II-restricted CTLs.

Material and methods

Primary glial cultures

Adult human glial cell cultures were established from surgical brain specimens obtained from the temporal lobes of patients treated for intractable seizures. After dissecting the meninges, the tissue was washed in phosphate-buffered saline (PBS), minced into 2–3 mm pieces, and treated with 0.2% trypsin and 20 μg/ml DNase (Sigma) in a volume of 10 ml for 40 min at 37°C. The tissue was vortexed every 10 min for 1 min. The trypsin digestion was stopped by adding 10% fetal calf serum (FCS). The cell suspension was then transferred to a 25 cm^2 tissue culture flask or onto coverslips precoated with poly-D-lysine (10 μg/ml) (Sigma) and left undisturbed for 1 h in a CO_2 incubator. Dulbecco's modified Eagle's medium (DMEM) containing 15% heat-inactivated FCS, glutamine, Hepes buffer, MEM nonessential amino acids, MEM vitamins, and penicillin/streptomycin was then added to the cells. Medium was changed every 48 h for the first two changes, then every 4 days.

Glioblastoma multiforme cell lines

Glioblastoma multiforme cell lines U-251 MG and U-373 MG were a gift from Dr. Darrel Bigner (Duke University, NC, U.S.A.) and the characteristics of these cell lines have been previously described (Bigner et al., 1981; Wikstrand et al., 1985). U-251 MG is an established cell line derived from a human glioblastoma multiforme. This cell line continues to produce glial fibrillary acidic protein (GFAP) in culture which is suggestive of its glial origin. U-251 MG expresses high levels of class I HLA molecules but not class II (DR) molecules. U-373 MG is another established cell line derived from a human glioblastoma multiforme but does not produce GFAP. This cell line expresses both class I and class II (DR) HLA molecules.

Immunofluorescence microscopy

Cells grown on coverslips were washed with PBS and incubated with anti-HLA class I (W6/32), anti-HLA class II-DR (L243) or nonimmune hybridoma supernatant. The cells were then washed twice and a fluorescein-conjugated sheep anti-mouse IgG (Kappel) was applied. After two washes, the coverslips were fixed in 2% paraformaldehyde for 15 min followed by Triton X-100 (0.1%) treatment for 5 min. Rabbit antiserum to GFAP (Dako, CA, U.S.A.) or normal rabbit serum was then applied (1:250 dilution), followed by tetramethylrhodamine B isothiocyanate (TRITC)-conjugated goat anti-rabbit antibody (Sigma). All antibody incubations were carried for 45 min at 25°C. The specificity of the immunostaining was established by the negative results obtained when one of the primary antibodies was omitted or nonimmune serum was applied. Elimination of either sheep anti-mouse or goat anti-rabbit antiserum resulted in the appropriate lack of specific staining indicating absence of interspecies cross-reactivity. Cells were examined on a Leitz fluorescent microscope and photographed with Kodak Ektachrome p800/1600 film.

Generation of cytotoxic T-cells

Influenza CTL are predominantly restricted by HLA class I molecules (Biddison, 1982). These were used to examine class I-restricted antigen presentation by human glial cells. Influenza virus-specific CTL were generated as described (Dhib-Jalbut et al., 1989). Briefly, 4×10^6 peripheral blood lymphocytes (PBL) matched with the primary glial cell targets for class I HLA molecules were cultured with influenza virus (A/JAP) for 1 h in 2 ml RPMI 1640 supplemented with glutamine in a 24-well tissue culture plate. Five percent human AB serum was then added and the cultures were carried for 7 days in a 5% CO_2 humidified incubator. Generation of influenza virus-specific CTL lines Q115 and 1D3 has been previously described (Cowan et al., 1987; Nuchtern et al., 1989). The line Q115 recognizes a synthetic peptide (M1) which corresponds to amino acid sequences 55–73 of the matrix protein of influenza virus A/JAP, and is restricted by HLA-A2 molecules. The line 1D3 recognizes influenza virus A/JAP and is restricted by HLA-A3 molecules.

MBP-specific T-cell lines were generated from DR2 homozygous donors by repeated stimulation of PBL with MBP (10 μg/ml) in the presence of autologous irradiated feeders (6000 rad) in RPMI 1640 media containing 10% human AB serum. After two passages, 10% human T-cell growth factor (Cellular Products, Buffalo, NY, U.S.A.) was added to the cultures. These lines were $CD4^+$ by FACS analysis, and DR2 restricted as determined by lysis of DR2-transfected targets (Jaraquemada, D. et al., in preparation). These lines were used 6 days after stimulation to examine presentation of class II-restricted antigen by glial cell targets.

Generation of targets

Glial cell targets obtained from primary cultures were treated with IFN-γ (100 units/ml, Genzyme, MA, U.S.A.) for 3 days and then infected with influenza virus (A/JAP) for 16 h. Targets obtained from the cell lines U-251 MG, U-373 MG, and an EBV-transformed B-cell line (K4B) were infected either with influenza virus A/JAP strain or influenza virus B/AA strain or pulsed with the M1 peptide (5 μg/ml) for 16 h. MBP targets were pulsed with human MBP (100 μg/ml) for 16 h. Glial cells were removed from the tissue culture flasks by trypsinization and washed with RPMI 1640 containing 5% FCS. The targets were then suspended in 0.3 ml medium containing 100 μCi/ml of $Na[^{51}Cr]O_4$ (New England Nuclear, Boston, MA, U.S.A.) and incubated for 90 min in a 37°C water bath. The chromated targets were then washed twice with medium and counted.

CTL assay

Varying numbers of effector cells were cultured with 5000 ^{51}Cr-labelled targets in triplicate wells of a 96-well microtiter plate in a volume of 200 μl of DMEM containing 10% FCS supplemented with glutamine. After 4 h of incubation in a humidified, 5% CO_2 atmosphere, the plates were spun at 5000 rpm for 5 min, the supernatants were harvested and ^{51}Cr release measured using a gamma counter. Percent target lysis was calculated as (experimental release − spontaneous release)/(detergent release − spontaneous release) × 100.

Results

Characterization of cell types in the primary cultures

Cells cultured on coverslips were examined 2–3 weeks after tissue dissociation. 70–80% of the cells used in this study were astrocytes as determined by staining with rabbit antiserum to GFAP and visualization by immunofluorescence microscopy. The majority of the remaining cells reacted with the monocyte/macrophage marker (leu-M3) and less than 5% reacted with rabbit antiserum to fibronectin. Cells that reacted with leu-M3 had morphological characteristics consistent with microglia including irregular rod-like shapes and short branching processes. The presence of oligodendrocytes was not examined in the cultures used in this study. However, examination of subsequent cultures established from different brains under similar conditions indicated the presence of less than 5% of cells that react with antibodies to the oligodendrocyte markers galactocerebroside (gift from Dr. M. Dubois-Dalcq) and MBP (Dakopatts). Cells reacting with antibodies to Von Willebrand factor (an endothelial cell marker) were

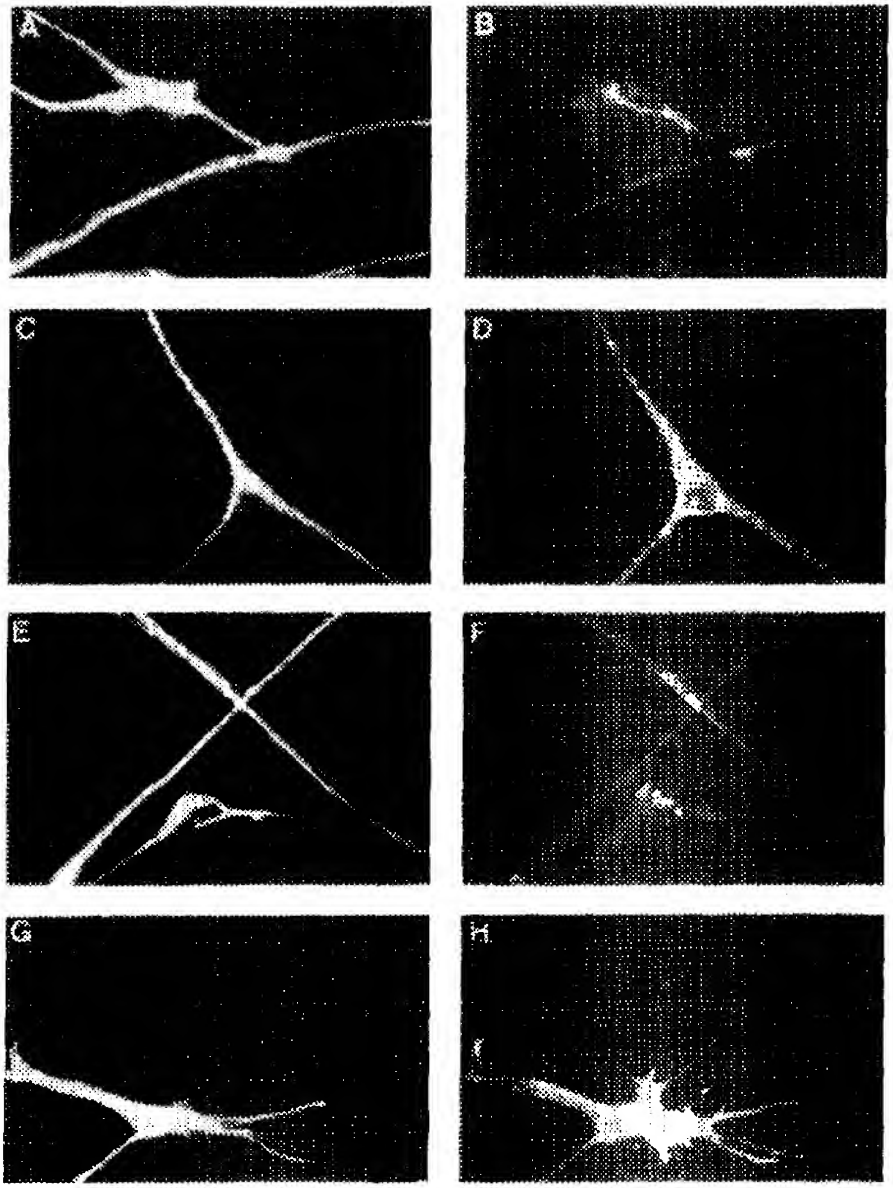

Fig. 1. Two-color immunofluorescence for GFAP (*A*, *C*, *E*, and *G*) and either class I or class II (DR) pre- and post-treatment with IFN-γ for 3 days. (*A* and *B*): Untreated cells costained for GFAP and HLA class I; (*C* and *D*): IFN-γ-treated cell contained for GFAP and HLA class I; (*E* and *F*): untreated cells costained for GFAP and HLA-DR. (*G* and *H*): IFN-γ-treated cells costained for GFAP and HLA-DR (×400).

not observed in these cultures. Approximately 50% of the GFAP-positive cells constitutively expressed low levels of HLA class I molecules (Fig. 1*A* and *B*) and less than 5% expressed HLA class II (DR) molecules (Fig. 1*E* and *F*), but expression could be upregulated on these cells up to 90% for both class I and class II DR by treatment with IFN-γ 100 units/ml for 3 days (Fig. 1*C*, *D*, and *G*, *H*). Upregulation of HLA class I and DR molecules was also observed on cells with a morphological appearance of macrophage/microglia.

Presentation of target antigens by primary cultures

The capacity of primary adult human glial cells (70% of which were GFAP positive) to present influenza A virus antigen to influenza virus-

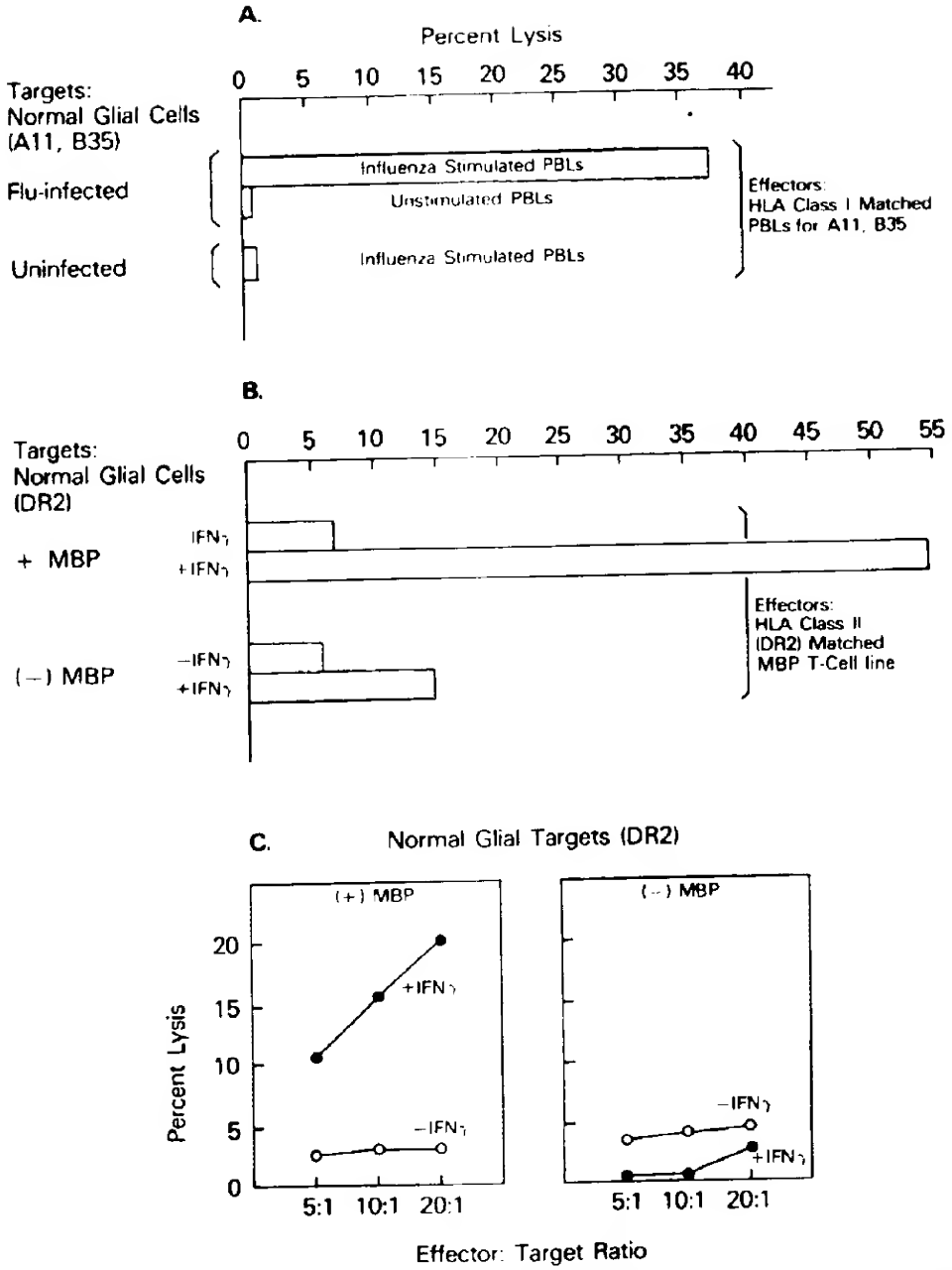

Fig. 2. (*A*) Lysis of primary adult human glial cells infected with influenza A/JAP virus by HLA class I-matched influenza-stimulated PBL from a healthy donor at effector to target ratio of 40 : 1. (*B*) Lysis of primary adult human glial cells obtained from an HLA DR2-positive individual by an MBP-specific DR2-restricted T-cell line at an effector to target ratio of 40 : 1. (*C*) Lysis of the same targets as in (*B*) with MBP-specific DR2-restricted T-cell line from a different donor at serial effector to target cell ratios.

specific class I HLA-restricted CTLs was examined. Following treatment with IFN-γ and subsequent infection with influenza A/JAP virus these glial cells were lysed by influenza virus-stimulated, HLA class I-matched PBL from a healthy donor. Lysis was not observed with the unstimulated PBLs or with the uninfected targets (Fig. 2*A*).

Lysis of MBP-pulsed targets by MBP-specific T-cell lines was used to examine HLA class II-restricted lysis. The MBP T-cell lines used in this

study are $CD4^+$ cells and are restricted by HLA class II molecules (Martin, R. et al., in preparation). Primary adult human glial cells (80% of which were GFAP positive) were obtained from a patient with an HLA-DR2 haplotype. Following treatment with IFN-γ and MBP these cells could be effectively lysed by two human MBP-specific DR2-restricted T-cell lines (Fig. 2*B* and *C*). The lysis was dependent on treatment of the target cells with IFN-γ. No lysis was obtained with targets treated with IFN-γ but not exposed to MBP (Fig. 2*B*). Similar results were obtained with another MBP-specific DR2-restricted T-cell line from a different donor at serial effector to target ratios (Fig. 2*C*).

Presentation of target antigens by $GFAP^+$ cells derived from glioblastoma multiforme

The primary adult human glial cell cultures also included nonastrocytic cell types, and therefore, the magnitude of ^{51}Cr release attributable to each cell population cannot be ascertained. Thus, the cell line U-251 MG which produces GFAP was used as a target in the subsequent experiment to establish that similar results could be obtained with cells presumably of glial origin. Moreover, the capacity of this glial target to be lysed by CTL in an HLA-restricted fashion was also examined.

U-251 MG cell line shares with normal adult astrocytes two relevant characteristics: it is GFAP positive and constitutively expresses HLA class I (A2) but not DR molecules. In contrast, U-373 MG does not express GFAP but expresses both HLA class I (A3) and DR molecules. U-251 MG was examined for its ability to present target antigens to the influenza virus-specific HLA-A2-restricted T-cell line Q115. An HLA-A2-positive EBV-transformed B-cell line (K4B) was used as a control target. As shown in Fig. 3, Q115 lysed U-251 MG and B-cell targets that were infected with the A/JAP strain of influenza virus (Fig. 3*A* and *I*) or pulsed with the M1 peptide (Fig. 3*B* and *J*) but not targets infected with a different strain of influenza virus (B/AA) (Fig. 3*C* and *K*) or uninfected targets (Fig. 3*D* and *L*). Lysis of U-251 MG target with the mismatched effectors (1D3) was not observed (Fig. 3*A–D*) indicating that the lysis of this target was restricted by HLA-A2 molecules. Similarly, no lysis was obtained

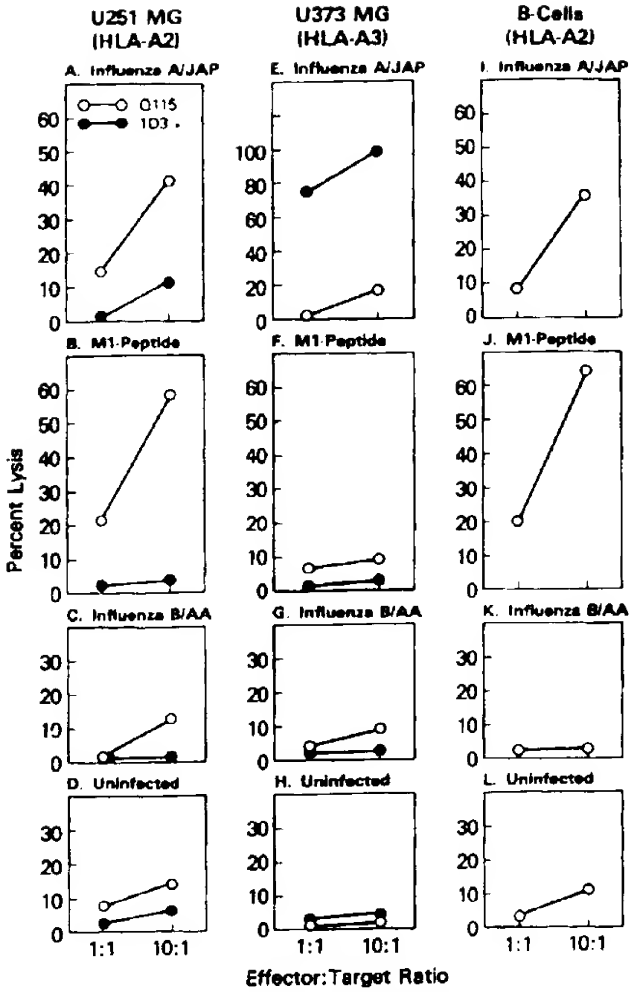

Fig. 3. Lysis of $GFAP^+$ glioblastoma multiforme cells (U-251 MG) by influenza virus-specific HLA-A2-restricted T-cell line Q115 (open circles). U-251 MG and EBV-transformed B-cells express HLA-A2. When infected with influenza virus, A/JAP strain or pulsed with the influenza virus M1 peptide, these targets were lysed by Q115 (*A*, *B*, *I*, *J*). No lysis was observed with targets infected with a different strain of influenza virus (B/AA) (*C*, *K*) or left uninfected (*D*, *L*). Glioblastoma U-373 MG targets which express HLA-A3 and infected with influenza virus were not lysed by T-cell line Q115 but could be lysed by the HLA-A3-restricted T-cell line 1D3 (*E*). Similarly, T-cell line 1D3 did not lyse U-251 MG targets (*A*).

with Q115 on the mismatched target (U-373 MG, HLA-A3 positive) but this target could be lysed by 1D3 (Fig. 3*E–H*) indicating that the lysis by these cell lines is HLA restricted.

Discussion

In this study, primary glial cell cultures were established from adult human brain. These cultures consisted primarily of astrocytes (70–80%) and microglia/macrophages (15–20%). The microglia/macrophage cells are likely to be derived from the brain rather than from peripheral blood contamination, since peripheral blood monocytes from the same patient cultured under similar conditions were morphologically different from the brain-derived microglia/macrophages. Fibroblasts were rarely detected in cultures less than 3 weeks old, but they increased in number, thereafter. Consequently, 2- to 3-week-old cultures were employed in order to study antigen presentation by glial cells. Under basal conditions, 50% of $GFAP^+$ cells expressed class I HLA molecules but only 5% expressed class II molecules. Both fluorescence intensity and number of cells expressing HLA molecules were increased by IFN-γ treatment. In general, these findings are in agreement with the observations reported in previous studies (Hirayama et al., 1986; Grenier et al., 1989) and personal communication with Dr. J.P. Antel.

The capacity of primary glial cells expressing class I and class II HLA molecules to present target antigens to CTL was examined. Since both viral and auto-antigens have been implicated in the development of autoimmune disease (Fontana et al., 1987), presentation of these antigens by primary human glial cells was studied. The present data suggest that primary mixed glial cell cultures can function as targets for antigen-specific CTL. As shown in Fig. 2*B* and *C*, the ability of these cells to function as targets for MBP CTL is dependent on treatment of the targets with IFN-γ which results in upregulation of HLA molecules. IFN-γ also has been shown to upregulate the expression of adhesion molecules such as intercellular adhesion molecule 1 (ICAM-1) on murine astrocytes which could contribute to more effective lysis of these cells (Frohman et al., 1989). Since the primary adult human glial cells contained nonastrocytic cell types such as microglia, the extent of ^{51}Cr release attributable to each cell population was uncertain. Therefore, either astrocytes or microglia or both could have functioned as targets for CTL. Attempts to separate the astrocytes and microglial cells have been hampered by the limited number of cells that can be obtained from adult brain tissue.

For these reasons an established human glioblastoma cell line U-251 MG which produces GFAP was used as targets to establish that similar results could be obtained with cells that are likely to be derived from a glial origin. The study performed with this cell line indicated that U-251 MG can process and present viral antigens as efficiently as B-cells, since both targets produced comparable lysis with the influenza virus and the M1 peptide (which does not require processing). Moreover, the results obtained with the U-251 MG targets indicated that the lysis of these targets is antigen specific and HLA restricted.

In autoimmune disease of the CNS the evidence that native cells of the CNS can present antigen in situ or become targets for the cellular immune response is largely circumstantial. Such evidence is derived from the immunohistochemical demonstration of class II HLA molecules on astrocytes, microglia, and endothelial cells in lesions of brains affected with multiple sclerosis or EAE (McCarron et al., 1985; Hayes et al., 1987; Traugott, 1987; Grenier et al., 1989). Studies addressing the functional capacity of these CNS cells to present antigen to T-cells have been conducted in murine systems and have largely focused on MBP (Fontana et al., 1984; McCarron et al., 1985; Sun and Wekerle, 1986). The present study suggests that adult human astrocytes and/or microglia can present viral or auto-antigen to cytotoxic T-cells. In addition, the results obtained with the $GFAP^+$ cell line U-251 MG support a role for astrocytic cells in presenting antigen to T-cells. These findings have implications for pathogenic mechanisms involved in the development of autoimmune diseases of the CNS. HLA molecules induced on glial cells by IFN-γ released during inflammation could render these cells capable of presenting antigen to T-cells. Glial cells could then play a role in the induction of an immune response within the CNS or become targets for CTL.

In addition, clearance of a virus infection from the brain may be dependent, in part, on CTL lysis of infected cells (Oldstone et al., 1986). This, in turn, is dependent on the upregulation of HLA molecules on the infected brain cells and the

capacity of these cells to process viral antigens. While this study suggests that adult human glial cells expressing class I HLA molecules may process and present viral antigen to HLA class I-restricted CTLs, it is not known whether similar processes occur in other cell types of the CNS. The persistence of certain viruses in the CNS may be related to a failure to upregulate HLA molecules, and subsequently, failure of CTL recognition of infected cells.

Finally, there is increasing interest in intracerebral transplantation of fetal tissue for patients with Parkinson's disease. Experimental evidence in rats suggests that microglia may participate in the immunological reaction to xenografts by functioning as antigen-presenting cells (Poltorak and Freed, 1989). Moreover, adult human mixed glial cell cultures have been shown to induce an allogeneic T-cell response (Grenier et al., 1989). The present findings suggest that human glial cells potentially can participate in the immunological reaction to intracerebral transplants under conditions that would lead to upregulation of HLA molecules.

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Transforming Growth Factor-β Inhibits Interferon-γ Secretion by Lymphokine-activated Killer Cells Stimulated with Tumor Cells

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Abstract

The effect of transforming growth factor-β (TGF-β) secreted by glioblastoma (T98G) cells on the secretion of interferon-γ (IFN-γ) by lymphokine-activated killer (LAK) cells stimulated with tumor cells was investigated in cocultures of LAK and Daudi cells supplemented with T98G culture supernatant, T98G culture supernatant preincubated with anti-TGF-β1 and anti-TGF-β2 neutralizing antibodies, anti-TGF-β1 and anti-TGF-β2 antibodies, or natural human TGF-β1 or recombinant human TGF-β2. LAK cells were incubated with anti-TGF-β1 and anti-TGF-β2 antibodies, and with T98G cells of which the supernatant contained both active and latent forms of TGF-β1 and TGF-β2, with or without neutralizing antibodies. Addition of the supernatant from T98G cells to LAK/Daudi culture caused inhibition of IFN-γ secretion by LAK cells. The inhibition was abolished by pretreatment of the supernatants with anti-TGF-β antibodies. Addition of TGF-β1 and TGF-β2 to the LAK/Daudi culture inhibited IFN-γ secretion by LAK cells in a dose-dependent manner. Addition of anti-TGF-β antibodies to the LAK culture resulted in increased IFN-γ secretion. T98G cells failed to stimulate LAK cells to secrete more IFN-γ. Addition of anti-TGF-β antibodies to the LAK-T98G culture resulted in increased IFN-γ secretion by LAK cells. These results suggest that most malignant glioma cells which secrete high levels of TGF-β can inhibit IFN-γ secretion by LAK cells even after tumor cell stimulation.

Key words: transforming growth factor-β, glioblastoma, interferon-γ, lymphokine-activated killer cell

Introduction

Malignant glioma cells secrete or express several cytokines, including transforming growth factor-β (TGF-β) which has immunosuppressive properties such as inhibition of T and B cell proliferation,[12,13] interleukin-2 receptor induction,[13] cytokine production,[5,6] natural killer cell activity,[26] cytotoxic T lymphocyte development,[25] lymphokine-activated killer (LAK) cell generation,[9] and production of tumor-infiltrating lymphocytes.[14] Most cells secrete TGF-β in a latent form,[29] which only manifests its biological functions after conversion to an active form.[30] Our previous studies showed that malignant glioma cells secrete both TGF-β1 and TGF-β2, and could convert TGF-β from a latent to an active form.[23,28] TGF-β secretion from malignant glioma cells may be involved in the immunosuppressive state of patients.[14,16]

Patients with malignant glioma have been treated with LAK cells.[19,24,33] Although LAK therapy had some efficacy against malignant gliomas, LAK therapy *in vivo* has been less effective than suggested by *in vitro* studies. This discrepancy may be due to gliotic tissue limiting the direct contact of LAK cells with tumor cells, the inability of LAK cells to infiltrate into all tumor tissue, or the effects of immunosuppressive cytokines such as TGF-β secreted by malignant glioma cells. LAK cells have a non-specific killing activity against tumor cells[8] and also secrete interferon-γ (IFN-γ) and tumor necrosis factor (TNF) when stimulated with tumor cells.[4] However,

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our previous study showed that cytokine(s) secreted by malignant glioma cells inhibit such secretion of TNF-α, TNF-β, and IFN-γ,[20,21] and that TGF-β suppressed TNF-α and TNF-β secretion by LAK cells even after stimulation with tumor cells.[22] This may occur in malignant gliomas, since most malignant glioma cells have the potential to activate latent TGF-β.[23,28] TGF-β also inhibits IFN-γ secretion by LAK cells.[2,5] However, the effect of TGF-β on IFN-γ secretion by LAK cells after stimulation with tumor cells has not been examined.

The present study investigated the effect of TGF-β secreted by glioblastoma cells on the secretion of IFN-γ by LAK cells stimulated with tumor cells.

Materials and Methods

I. Cell lines

Human glioblastoma cell line (T98G) and Daudi cell line (human B tumor cell line derived from a patient with Burkitt's lymphoma) were obtained from the Japanese Cancer Research Resources Bank (Tokyo). These tumor lines were maintained in RPMI1640 medium supplemented with 2 mM glutamine, 100 μg/ml kanamycin, 0.05 mM 2-mercaptoethanol, and 10% fetal bovine serum.

II. Induction of LAK cells

LAK cells were induced as described elsewhere.[21] Briefly, peripheral blood mononuclear cells were collected from the heparinized peripheral blood of normal healthy donors by centrifugation on a Ficoll-Paque (Pharmacia Fine Chemicals, Uppsala, Sweden) density gradient. Peripheral blood mononuclear cells were cultured at 2×10^6 cells/ml in RPMI1640 medium with 10% fetal bovine serum and 2000 Japan Reference units/ml of interleukin-2 (Takeda Pharmaceutical Co., Ltd., Osaka). After culturing for 3 to 5 days, LAK cells were collected.

III. Collection of conditioned medium

Subconfluent T98G cells were cultured in tissue culture flasks (25 cm^2; Becton Dickinson, Franklin Lakes, N.J., U.S.A.), washed three times with phosphate-buffered saline, and cultured in 3 ml of serum-free RPMI1640 medium. After culturing for 24 hours, the supernatants were collected, filtered (0.22 mm; Millipore Ltd., Tokyo), and stored at -20°C until use. Previous studies indicated that the supernatants contained both active and latent forms of TGF-β1 and TGF-β2.

IV. LAK cell culture with Daudi cells

LAK cells were adjusted to a concentration of 1×10^6 cells/ml in complete medium with 5% fetal bovine serum and incubated with Daudi cells at a LAK/Daudi cell ratio of 10:1 at 37°C under 5% CO_2 in 24-well plates (Corning, Tokyo). Various LAK/Daudi cultures were supplemented with 1) 300 μl of T98G culture supernatant, 2) 600 μl of T98G culture supernatant preincubated with anti-TGF-β1 (R&D Systems, Minneapolis, Minn., U.S.A.) and anti-TGF-β2 (R&D Systems) neutralizing antibodies (final concentration 6 μg/ml), 3) anti-TGF-β1 and anti-TGF-β2 antibodies (final concentration of each antibody 6 μg/ml), and 4) 3 ng, 300 pg, 30 pg, or 3 pg/ml of natural human TGF-β1 (Genzyme, Cambridge, Mass., U.S.A.) or recombinant human TGF-β2 (Austral Biologicals, San Ramon, Cal., U.S.A.) in a total volume of 1 ml. LAK cells were incubated with anti-TGF-β1 and anti-TGF-β2 antibodies (final concentration of each antibody 6 μg/ml) and chicken immunoglobulin G (Cappel, Durham, N.C., U.S.A.) or rabbit immunoglobulin G (Cappel) (final concentration 6 μg/ml). After culturing for 16 to 18 hours, the culture supernatants were harvested, filtered, and stored at -20°C until use.

Preliminary studies indicated that at least 10 μg/ml of anti-TGF-β1 or anti-TGF-β2 antibodies completely neutralized 2.5 ng/ml of natural human TGF-β1 or recombinant human TGF-β2, respectively, and that anti-TGF-β1 antibody did not neutralize TGF-β2 and *vice versa*. Therefore, the T98G culture supernatant was preincubated with anti-TGF-β1 (10 μg/ml) and anti-TGF-β2 (10 μg/ml) antibodies for 1 hour at room temperature. We used Daudi cells, since Daudi cells are commonly used as a tumor target of LAK cells and are resistant to natural killer cell-mediated cytotoxicity. Furthermore, no TGF-β activity was detected by bioassay in the supernatant from Daudi cell culture.

V. LAK cell culture with T98G cells

LAK cells were adjusted to a concentration of 1×10^6 cells/ml in complete medium with 5% fetal bovine serum and incubated with T98G cells at a LAK/T98G cell ratio of 10:1 at 37°C under 5% CO_2 in 24-well plates (Corning). LAK/T98G cultures were supplemented with anti-TGF-β1 and/or anti-TGF-β2 antibodies. After culturing for 16 to 18 hours, the culture supernatants were harvested, filtered, and stored at -20°C until use.

VI. Measurement of IFN-γ activity

IFN-γ activity in the culture supernatants was measured by a modified bioassay described elsewhere.[21] Supernatants from LAK and tumor cells cultures contained TNF-α, TNF-β, and IFN-γ.[20,21]

Most supernatants from malignant glioma cells contained both latent and active forms of TGF-β1 and TGF-β2.[23,28] These cytokines may have antiproliferative activity against most cell types. However, T98G cells are resistant to the antiproliferative activity of recombinant human TNF-α, TNF-β, and TGF-β (data not shown). On the other hand, T98G cells are very sensitive to IFN-γ and the antiproliferative activity of IFN-γ against T98G cells is reproducible, with a coefficient of variation between assays of 4.8–24.3% depending on dilution. Therefore, we used T98G cells for the bioassay to detect IFN-γ activity.

Serial two-fold dilutions of each supernatant were made in duplicate in RPMI1640 medium supplemented with 10% fetal bovine serum in 96-well flat-bottomed microtiter plates. The T98G cells were seeded at 2000 cells/well, then incubated at 37°C under 5% CO_2. After 3 days of incubation, the cells were stained with 0.5% crystal violet in 20% methanol. The dye was eluted with 50% ethanol, and the absorbance at 570 nm was determined by a Titertek Multiskan (Flow Laboratories, Inc., Helsinki, Finland). The relative percentage viability was calculated as: (optical density [supernatant-treated]/optical density [untreated]) × 100. Recombinant human IFN-γ (Toray Co., Ltd., Tokyo) with a specific activity of 4.2×10^7 IU/mg was used as an internal standard. The supernatants were preincubated with anti-TNF-α (5 μg/ml; R&D Systems) or anti-IFN-γ (10 μg/ml; Genzyme) to specify the activity. In preliminary studies, the antibodies completely neutralized 1 ng/ml of recombinant human TNF-α (Dainippon Pharmaceutical Co., Ltd., Osaka) and 1000 IU/ml of IFN-γ (Toray Co., Ltd.), respectively. The dilution causing growth inhibition of 50% of the cells was determined and the activity of IFN-γ (IU/ml) was calculated from the standard dilution curve.

Results

I. Inhibition of IFN-γ secretion by TGF-β in LAK/Daudi culture

LAK cells constitutively secreted IFN-γ, and Daudi cell stimulation resulted in the increase of IFN-γ secretion by LAK cells (Fig. 1). The levels of IFN-γ secreted by LAK cells were variable in different donors. The increased IFN-γ secretion by LAK cells was suppressed by addition of T98G culture supernatant to the LAK/Daudi culture. When the LAK/Daudi culture was supplemented with T98G culture supernatant pretreated with anti-TGF-β1 and anti-TGF-β2 neutralizing antibodies or with the antibodies alone, IFN-γ secretion by LAK cells

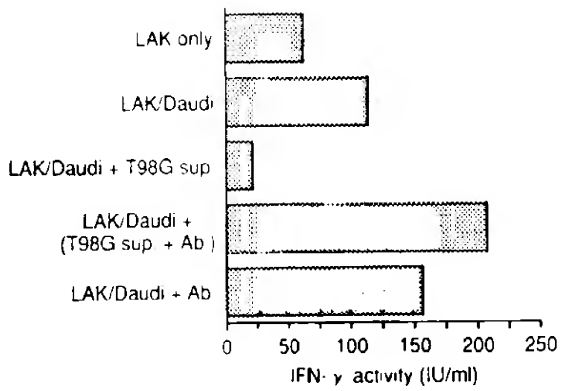

Fig. 1 Effect of T98G culture supernatant on IFN-γ secretion of LAK cells stimulated by Daudi cells. Cocultures of LAK and Daudi cells were supplemented with T98G culture supernatant to 1 ml total volume. Ab.: anti-TGF-β1 (20 μg/ml) and anti-TGF-β2 (20 μg/ml) antibodies, T98G sup.: T98G culture supernatant, T98G sup. + Ab.: T98G culture supernatant preincubated with anti-TGF-β1 and anti-TGF-β2 antibodies. The figure represents three experiments.

achieved a nearly two-fold increase compared to that in LAK/Daudi culture. IFN-γ activity in the supernatants was specified using anti-IFN-γ neutralizing antibody. The addition of anti-IFN-γ antibody almost completely abolished the growth inhibitory activity in the supernatants. On the other hand, the addition of anti-TNF-α antibody did not affect the activity.

II. Effect of anti-TGF-β antibodies on IFN-γ secretion by LAK cells

When T98G supernatant pretreated with anti-TGF-β antibodies was added to LAK/Daudi culture, IFN-γ secretion by LAK cells increased more than in LAK/Daudi culture (Fig. 1). Therefore, anti-TGF-β neutralizing antibodies were added to LAK cell culture to examine how anti-TGF-β alone affects IFN-γ secretion by LAK cells. IFN-γ secretion by LAK cells showed a three- to nine-fold increase with the addition of anti-TGF-β1 (chicken immunoglobulin G) and/or anti-TGF-β2 (rabbit immunoglobulin G) neutralizing antibodies, but was minimal with the addition of chicken and rabbit immunoglobulin G (Fig. 2). This indicates that LAK cells themselves may secrete low levels of TGF-β1 and TGF-β2, and can convert TGF-β1 and TGF-β2 from the latent to active forms.

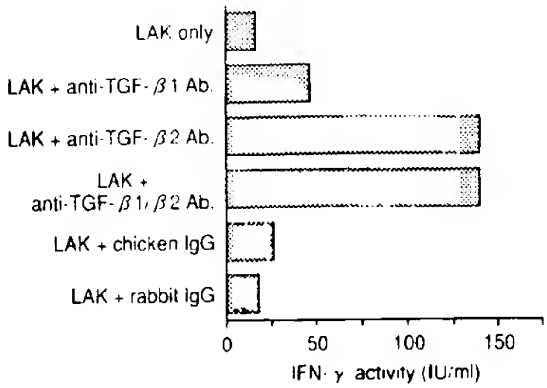

Fig. 2 Effect of anti-TGF-β1 and anti-TGF-β2 antibodies on IFN-γ secretion by LAK cells. Anti-TGF-β1 (chicken immunoglobulin G 6 μg/ml) and/or anti-TGF-β2 (rabbit immunoglobulin G 6 μg/ml) antibodies were added to LAK culture. Ab.: antibody, IgG: immunoglobulin G. The figure represents two experiments.

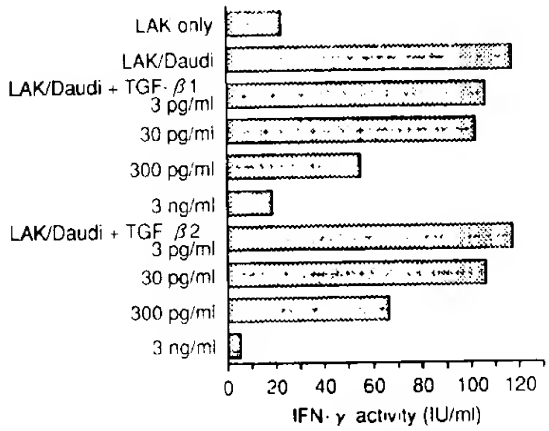

Fig. 3 Effect of TGF-β1 and TGF-β2 on IFN-γ secretion by LAK cells stimulated by Daudi cells. Natural human TGF-β1 or recombinant human TGF-β2 was added to LAK/Daudi culture. The figure represents two experiments.

III. Effect of exogenous TGF-β on IFN-γ secretion by LAK cells

Natural human TGF-β1 and recombinant human TGF-β2 suppressed IFN-γ secretion by LAK cells in LAK/Daudi culture in a dose-related manner (Fig. 3). Addition of 300 pg/ml of TGF-β1 or TGF-β2 resulted in around 50% inhibition of IFN-γ secretion by LAK cells stimulated with Daudi cells. Furthermore, addition of 3 ng/ml of TGF-β1 or TGF-β2 resulted in complete inhibition of increased IFN-γ secretion by LAK cells.

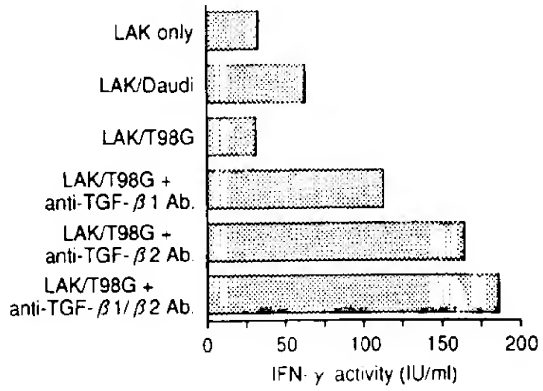

Fig. 4 Effect of anti-TGF-β1 and anti-TGF-β2 antibodies on IFN-γ secretion by LAK cells stimulated with glioblastoma cells. Ab.: antibody. The figure represents two experiments.

IV. Inhibition of LAK cell IFN-γ secretion by T98G cells

IFN-γ secretion by LAK cells was not stimulated by T98G cells in contrast to the stimulation by Daudi cells (Fig. 4). However, the addition of anti-TGF-β1 and/or anti-TGF-β2 antibodies to the LAK/T98G culture resulted in increased IFN-γ secretion by LAK cells. Preliminary studies[23] found the T98G culture supernatant contained more than 1 ng/ml of total active forms of TGF-β1 and TGF-β2 in several assays where recombinant TGF-β2 was used as an internal standard.

Discussion

The present study indicated that TGF-β secreted and activated by glioblastoma cells inhibited IFN-γ secretion by LAK cells even after tumor cell stimulation, and that exogenous TGF-β1 and TGF-β2 also inhibited IFN-γ secretion by LAK cells. Together with our previous results,[22] this suggests that TGF-β may inhibit the secretion of TNF-α, TNF-β, and IFN-γ by LAK cells stimulated with tumor cells. The present study provided additional evidence that LAK cells may secrete low levels of TGF-β1 and TGF-β2, and can convert TGF-β1 and TGF β2 from the latent to active forms. Although TGF-β in the culture media was derived from both glioblastoma cells and LAK cells, the present results suggest that glioblastoma-derived TGF-β mainly inhibited IFN-γ secretion by LAK cells, which is consistent with our previous study.[22] Recent reports have shown that interleukin-2 stimulation of peripheral blood mononuclear cells

results in up-regulation of intracellular TGF-β messenger ribonucleic acid and TGF-β biologic activity secreted in culture media,[11,13] and that peripheral blood mononuclear cells secrete and activate latent TGF-β1 on interleukin-2 stimulation.[15] Therefore, the activation of latent TGF-β1 and TGF-β2 by LAK cells may function as a negative feedback mechanism for cytokine secretion. However, activation of TGF-β by LAK cells apparently cannot inhibit efficiently the secretion of TNF-α, TNF-β, and IFN-γ by LAK cells stimulated with Daudi cells. This suggests that most malignant glioma cells which secrete high levels of TGF-β can inhibit IFN-γ secretion by LAK cells even after tumor cell stimulation. On the other hand, the low level of TGF-β secreted and activated by LAK cells may not be sufficient to inhibit IFN-γ secretion by LAK cells after stimulation with tumor cells.

The present study used glioblastoma cells with a supernatant containing both active and latent forms of TGF-β1 and TGF-β2[23] for the coculture of LAK and tumor cells. Some tumor cells can convert TGF-β from a latent to an active form,[31] although the mechanism of the activation is not well understood. LAK cells injected into patients with tumors may secrete more TNF-α, TNF-β, and IFN-γ, as well as kill tumor cells, if the LAK cells contact the tumor cells. However, LAK cells may fail to secrete more cytotoxic cytokines when in contact with tumor cells which can convert latent TGF-β to the active form, such as malignant gliomas.[28] This inhibition of cytokine secretion by TGF-β may suppress the propagation of cytokine signals and therefore partly explain the low efficacy of LAK therapy and other immunotherapeutic modalities.[19,24,27,33]

Lymphocytes infiltrate into most tumor tissues, and some are cytotoxic against tumor cells after *in vitro* expansion.[17,18,32] However, activated lymphocytes express more high affinity TGF-β receptors than resting lymphocytes, TGF-β downregulates the expression of interleukin-2 receptors on T lymphocytes, and activated T cells synthesize and secrete TGF-β, which inhibits interleukin-2-dependent T cell proliferation.[13] Therefore, the presence of active TGF-β in tumor tissue may paralyze the function of these tumor-infiltrating lymphocytes by inhibiting cytokine secretion (TNF-α, TNF-β, and IFN-γ) and by inhibiting proliferation through TGF-β receptors.

An active form of TGF-β is produced by cocultures of vascular endothelial cells and pericytes.[1] Furthermore, vascular endothelial cells have tissue plasminogen activator activity,[7] and latent TGF-β is converted to an active form by plasmin.[3] Most malignant cells are rich in blood vessels, so conversion of TGF-β from a latent to an active form may occur around the vascular endothelial cells through which lymphocytes migrate into tumor tissue. Therefore, latent TGF-β secreted by tumor cells may be converted to an active form by the tumor cells themselves and by the vascular endothelial cell-pericyte complex, resulting in a combination of poor conditions under which activated lymphocytes infiltrating into tumor tissue must attack tumor cells.

In conclusion, tumor cells which mostly secrete a high level of TGF-β and can convert TGF-β from a latent to an active form may inhibit the secretion of cytotoxic cytokines (TNF-α, TNF-β, and IFN-γ) by activated lymphocytes even after tumor cell stimulation. In addition, TGF-β secreted by tumor cells may suppress the propagation of the immune reaction by inhibiting the secretion of the cytokines by activated lymphocytes infiltrated into tumor tissue. Methods to downregulate TGF-β secretion from malignant glioma cells are potentially an effective treatment modality for patients with malignant tumors. Currently, the therapeutic reduction of TGF-β activity is the focus of study in cancer patients,[9,10] and further studies to elucidate the mechanism of regulation of TGF-β secretion and activation by malignant tumor cells are needed.

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Commentary

Dr. Naganuma and colleagues present a well constructed experimental study investigating the pivotal role of cytokines in the development and spread of glioblastomas. The hypothesis tested in this work is that glioblastoma cells produce an active form of TGF-β which plays a major role in the immunosuppression seen in patients with glioblastoma. Specifically, this research shows that the active TGF-β secreted by T98G glioblastoma cells is capable of inhibiting interferon-γ release from stimulated LAK cells. In addition to providing a scientific basis for the observed lack of clinical effect of LAK cell therapy in patients with glioblastoma, this study may serve as a stimulus for enhancement of cytotoxic cytokine mediated treatment by blocking the effects of TGF-β. This type of hypothesis-driven methodical research may ultimately provide the key to effective therapy for malignant glioma.

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The authors have shown that transforming growth factor (TGF)-β secreted by glioma cells inhibited interferon (IFN)-γ secretion by lymphokine activated killer (LAK) cells. This finding is very interesting for the practice of clinical LAK therapy, providing evidence that TGF-β secretion by glioma cells can inhibit the antitumor effect of LAK cells. The response of gliomas to clinical LAK therapy is variable and it is quite difficult to predict the sensitivity of each tumor to LAK therapy. We would like to know how many gliomas secreted higher levels of TGF-β or how high the TGF-β level was.

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Human glioblastoma cell line 86HG39 activates T cells in an antigen specific major histocompatibility complex class II-dependent manner

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Summary

The capacity of three different human glioblastoma cell lines to activate human T cells was analysed by measuring major histocompatibility complex (MHC) antigen expression, monokine secretion and lectin, mAb OKT3 and antigen-driven T cell proliferation. All glioblastoma cells tested were able to induce PHA and concanavalin A (ConA)-driven T cell proliferation in a dose-dependent fashion, while all failed to induce T cell activation with mAb OKT3. In addition, the glioblastoma cell line 86HG39 was able to induce tetanus toxoid and toxoplasma lysate antigen-specific T cell proliferation. The responding T cell lines originated from only one out of five different donors. This foreign antigen-specific T cell proliferation induced by 86HG39 cells could be inhibited with mAb L243 directed against HLA-DR molecules. The study of monokine secretion by 86HG39 cells showed a strong interleukin (IL)-6 secretion after lipopolysaccharide (LPS) treatment, whilst no IL-1 secretion was observed. Furthermore, only 86HG39 cells were positive for HLA-DR molecules, whereas interferon (IFN)γ treatment of 87HG28 and 87HG31 cells was necessary for the induction of class II antigen expression. Thus, cell line 86HG39 shows many features of an antigen presenting cell and the interaction of these cells with MHC compatible human T cells might be a useful model to study cellular immune reactions within the central nervous system.

Introduction

The initiation of cellular immune reactions within the central nervous system (CNS) is strictly controlled by the blood–brain barrier which consists of endothelial cells and astrocytes. Furthermore, only few cells expressing major histocompatibility complex (MHC) class II antigens are found within the normal brain parenchyma (Fontana et al., 1987). Inflammatory reactions within the CNS, however, lead to an at least partial destruction of the blood–brain barrier, and the amount of class II antigen-positive cells increased and large amounts of cytokines, for example interleukin (IL)-1, IL-6 and TNF, could be found within the cerebrospinal fluid (Waage et al., 1989).

Within the CNS, two types of accessory cells exist that are capable of antigen presentation: microglial cells and astrocytes. Microglial cells are possibly blood-derived cells, expressing Mac-1 in a similar fashion as peripheral accessory cells (Giulian, 1987). In contrast, astrocytes express glial fibrillary acidic protein (GFAP) and are of neuroepithelial origin. Astrocytes are also capable of functioning as accessory cells. For example it has been described that γ-interferon (IFNγ) leads to an induction of class II MHC antigen expression on astrocytes (Wong et al., 1984). In addition it was shown that murine IFNγ-treated astrocytes were

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able to present antigen to MHC-restricted preactivated T cells (Fierz et al., 1985). More recently, human astrocytes have been shown to present foreign antigen to class I- and class II-restricted human cytotoxic T cells (Dhib-Jalbut et al., 1990).

Human brain cells for the study of cellular immune reactions are difficult to obtain. Most of our knowledge about brain accessory cells has been derived from murine or rat model systems or obtained by the use of human brain tumor cells. Human glioblastoma cells have been shown to express many features of accessory cells, for example monokine secretion (IL-1, IL-6, TNF) and IFNγ-responsible class II antigen expression (Takiguchi et al., 1985). Also, the presentation of antigen to human cytotoxic T cells resulting in a MHC-restricted induction of cytotoxic effector function was described (Dhib-Jalbut et al., 1990). Furthermore, glioblastoma cells were responsible for regulatory signals provided by the monokines IL-1 and TNF (Lachman et al., 1987; Nishida et al., 1989). To our knowledge, no data are available on whether glioblastoma cells could activate human T cells in a foreign antigen-specific MHC-restricted fashion.

We investigated the antigen-presenting capacity of three recently described glioblastoma cell lines, 86HG39, 87HG28 and 27HG31. These three glioblastomas expressed GFAP in early in vitro passages within 50% of cells, and only 86HG39 cells partially expressed GFAP also after 100 in vitro passages, suggesting their glial origin (Bilzer et al., 1991). The aim of this study was the characterization of accessory cell functions of glioblastoma cells, involving class II antigen expression and regulation, IL-6 and IL-1 production and the capacity to provide co-stimulatory signals necessary for human T cell proliferation.

Materials and Methods

Chemicals and antibodies

Tetanus toxoid (TT) was purchased from Behringwerke AG, Marburg, Germany (purity 1490 Lf/mgN). *Toxoplasma gondii* strain BK was a kind gift of Drs. H.M. Seitz and M. Saathoff (Inst. für Med. Parasitologie, Bonn, Germany). Tachyzoites were maintained in vitro within L929 fibroblasts, purified from cell debris by subsequent washes, and used for antigen preparation. Toxoplasma antigen from freeze-killed tachyzoites was prepared as described (Hughes and Balfour, 1981).

Concanavalin A (ConA) was purchased from Pharmacia, Uppsala, Sweden, and phytohemagglutinin A (PHA) and mitomycin C were obtained from Sigma, Deisenhofen, Germany. Iscove's modified Dulbecco's medium (IMDM, Gibco, Grand Island, NY) was supplemented with 2 mM L-glutamine and 5% heat-inactivated FCS (56°C, 30 min), and was used as culture medium (CM) for all cell lines.

The hybridomas OKT11 (anti CD2), OKT3 (anti CD3), OKT4 (anti CD4), OKT8 (anti CD8), L243 (anti HLA-DR) and W6/32 (anti-HLA class I antigen) were all obtained from the 'American Type Culture Collection (ATCC, Rockville, MA). All antibodies were used as ascites diluted 1:500–1:3000.

Human rIL-1 and human rIFNγ was purchased from Genzyme, Cambridge, MA, Human rIL6 was from Pharma Biotechnologie (PBH, Hannover, Germany). Human rIL-2, used for T cell expansion in doses between 1–5 ng/ml, was a generous gift of Dr. L. Kaiser, EuroCetus, Frankfurt/M, Germany.

Human glioblastoma cell lines

Glioblastoma cell lines 86HG39, 87HG31 and 87HG28, which were recently described by means of immunocytochemical and morphological criteria, were obtained from Drs. T. Bilzer and W. Wechsler (Institut für Neuropathologie, Heinrich-Heine-Universität, Düsseldorf, Germany) (Bilzer et al., 1991). Cells were grown in CM in tissue culture flasks (Costar, Cambridge, MA) and divided weekly in ratios between 1:2 and 1:10 depending on the proliferation rate. In this study, cells between 70 and 150 in vitro passages were used. A culture time of more than 500 days excludes contaminating macrophages and microglia cells because they do not survive in culture medium. To simplify harvesting of glioblastomas, the cells were detatched with trypsin/EDTA and viability was tested by trypan blue exclusion after harvesting.

Establishment of human T cell lines

T cell lines were established as previously described (Däubener et al., 1987). In brief, PBMC obtained from five different donors by density gradient centrifugation on Ficoll-Hypaque (Pharmacia, Uppsala, Sweden) were stimulated with tetanus toxoid (TT, 3 μg/ml) or toxoplasma lysate antigen (TLA, 5 μg/ml). After 7–10 days culturing in vitro, cells were harvested and restimulated with antigen on irradiated (3000r) syngeneic EBV-transformed B cells and later on expanded by the addition of IL-2. In the case of cell lines from donor Hg, restimulation of cells was also done on mitomycin-treated 86HG39 cells and TT in parallel. Mitomycin treatment was done by incubation for 1 h of $3–10 \times 10^6$ 86HG39 cells within 1 ml CM containing 50 μg mitomycin. Restimulation of established lines was done every 8–14 days. In this research, T cells were used after a minimum of 1 month in vitro culture, and antigen specificity was controlled in every restimulation period. It was found that, independent of the type of accessory cell, TT-specific T cell lines did not respond to TLA and TLA-specific T cells did not respond to TT. All cell lines used were checked monthly to be free

of mycoplasma contamination by the cultivation method described earlier (Schmitt et al., 1988).

T cell proliferation assay

Antigen-specific T cells (0.5×10^5) were co-cultured in 96-well flat-bottom culture plates (Costar, Cambridge, MA) with mitomycin-treated glioblastoma cells (2×10^4–0.2×10^4) and either TT (3 μg/ml), TLA (5 μg/ml), PHA (0.5 μg/ml) or ConA (0.5 μg/ml) were added. For blocking experiments with mAbs, the antibodies were added to the glioblastoma cells as 1:500 diluted ascites 30 min prior to the addition of T cells and antigen. After 72 h incubation at 37°C the cultures were pulsed with 0.15 μCi of [^{3}H]thymidine (Amersham Buchler, Braunschweig, Germany) for 14–18 h and then processed for liquid scintillation counting. Results are expressed as mean cpm of triplicate or quadruplicate cultures ± SE. Each experiment shown is representative of minimally three similar experiments.

B9 bioassay for IL-6

2×10^5 glioblastoma cells were treated in 24-well culture plates (Costar, Cambridge, MA) for 24 h with different amounts of lipopolysaccharide (LPS) (Sigma, Deisenhofen, Germany) and the supernatant was removed and added in three-fold dilutions to 2.5×10^3 B9 cells. The proliferation of B9 cells was monitored by [^{3}H]thymidine uptake after 48–72 h culture in vitro. In each experiment, rIL-6 was used as positive control; one unit of IL-6 giving rise to half maximal proliferation. The B9 bioassay is described to be specific for IL-6 and no cross-reactions with other cytokines, especially IL-1, are known (Helle et al., 1988). B9 cells in our laboratory were maintained in CM containing 1% of supernatant of LPS-stimulated PBMC.

EL-4 / CTLL bioassay for IL-1

The EL-4/CTLL bioassay for IL-1 was done as described (Gearing et al., 1987). EL-4 cells expanded in CM, and CTLL cells in CM with 5 ng/ml rIL-2 were harvested and extensively washed prior to use in the bioassay. LPS-induced supernatants of glioblastoma cells were titrated in three-fold dilution, added to 3×10^4 EL-4 cells in a total volume of 200 μl culture medium and incubated for 24 h at 37°C. Then the culture supernatant was harvested and added to 2×10^3 CTLL cells and again incubated for 48 h. The cultures were pulsed with [^{3}H]thymidine for the final 16 h. All cytokine tests were done in triplicate, and rIL-1 was used as positive control. One unit of IL-1 gives rise to half maximal [^{3}H]thymidine uptake

HLA-antigen expression

5×10^5 glioblastoma cells were treated with mAbs L243 or W6/32 used as 1:1000 diluted ascites for 30 min. After extensive washing, a FITC-labelled F(ab')$_2$ fragment of goat anti-mouse Ig (Dianova, Hannover, Germany) was added for 30 min. After three washing steps, cells were analysed with a FACScan (Becton Dickinson, Mountain View, CA). For each histogram, 1×10^4 cells were counted. As a negative control, cells were stained with only the second step reagent.

Determination of HLA-phenotypes

A two-colour microcytotoxicity assay was employed for HLA class I and II serotyping as follows: mononuclear cell suspensions, Ficoll separated from blood, and cell lines from tissue culture were incubated with a panel of well-defined HLA sera encompassing the whole range of known alleles. After addition of fresh rabbit serum as a source of complement, ethidium bromide stained classified dead cells by red fluorescence, whilst living cells were identified by carboxyfluorescine diacetate as green cells.

HLA phenotyping was done by Dr. G. Kögler, Institut für Blutgerinnungswesen und Transfusionsmedizin, Heinrich-Heine-Universität Düsseldorf, Germany.

Results

MHC-antigen expression by glioblastoma cells

Antigen-presenting cells are defined by their capacity to express MHC class II antigens and to produce cytokines involved in T cell activation (Unanue, 1984). In order to investigate the accessory cell capacity of human glioblastomas, we first analysed the class I and class II antigen expression of the glioblastoma cell lines 86HG39, 87HG28 and 87HG31. The cells were incubated with mAb L243 against human class II monomorphic determinants or mAb W6/32 against human class I monomorphic antigens. After detection of antibodies by a goat anti-mouse FITC-labelled antibody, cells were analysed with FACScan flow cytometry.

Cell lines 87HG28 and 87HG31 expressed class I antigens (data not shown) but no class II antigens by flow cytometry. In contrast, 86HG39 cells were positive for both MHC antigens. Treatment of glioblastoma cells 87HG28 and 87HG31 with 100 U/ml IFNγ for 48 h led to a strong induction of class II antigen expression and virtually all cells became positive. Untreated 86HG39 cells were positive for class II antigens and IFNγ treatment led to an slightly enhanced HLA-DR expression, as shown in Fig. 1.

IL-6 and IL-1 production by human glioblastoma cells

Major monokines involved in T cell activation are IL-1 and IL-6. Thus, investigation of IL-6 production of 86HG39, 87HG28 and 87HG31 was performed after cultivation of the cells for 24 h with 0.01 to 1 μg/ml

LPS. Cell supernatant, measured by B9 assay, revealed that all glioblastomas tested were able to produce IL-6. Whilst 87HG28 and 87HG31 cells were found to secrete IL-6 spontaneously, 86HG39 cells produced IL-6 only after LPS treatment. Figure 2A shows data obtained with 86HG39 cells stimulated with 1 μg/ml LPS.

In contrast, the three glioblastomas did not secrete detectable amounts of IL-1 even after treatment with 10 μg/ml LPS. The supernatant of PBMC cells cultivated under the same conditions, used as a control, contained large amounts of IL-1. The EL-4/CTLL assay used for the detection of IL-1 is finally dependent on the IL-2 driven proliferation of CTLL cells (Gearing et al., 1987). Since it was described that several glioblastoma cells produce a factor which blocks IL-2-driven proliferation (Fontana et al., 1984), we investigated whether the failure of IL-1 detection was due to the action of this factor. Therefore rhu IL-1 was diluted with the supernatant of glioblastomas. As shown in Fig. 2B, IL-1 was detected in the supernatant of 86HG39 cells stimulated with 10 μg/ml LPS as well as in the control, indicating that no suppressive factor for IL-2-driven CTLL proliferation was produced by 86HG39 cells.

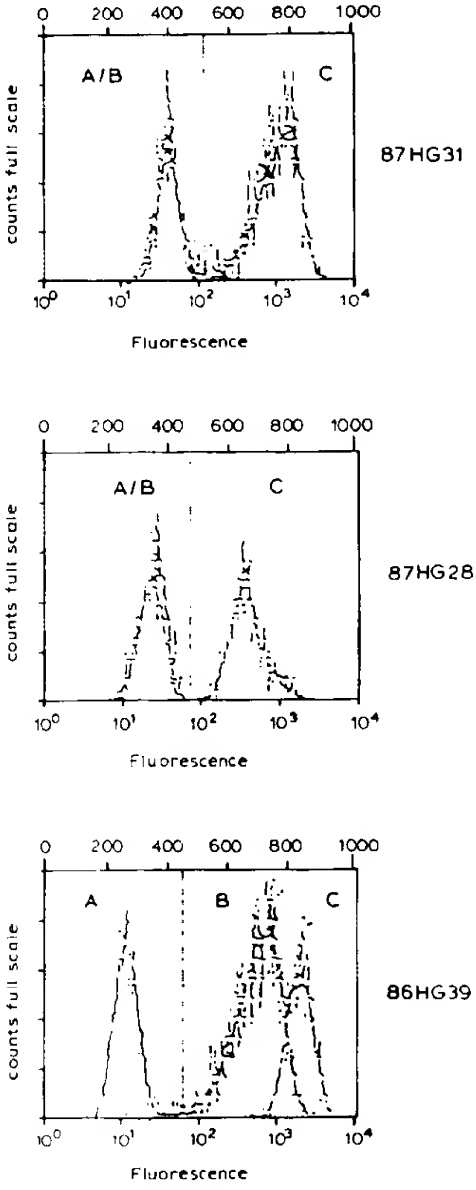

Fig. 1. Glioblastoma cells (B) or IFNγ-treated glioblastoma cells (C) were harvested, washed and incubated with 1:3000 diluted ascites containing mAb L243 directed against HLA-DR antigens. Binding of mAbs was detected by a FITC-conjugated goat anti-mouse $F(ab)_2$ fragment (1:50). In the control (A) background staining of glioblastoma cells only treated with the second step reagent is shown. Samples were analysed with FACScan flow cytometry and for each histogram 1×10^4 cells were counted.

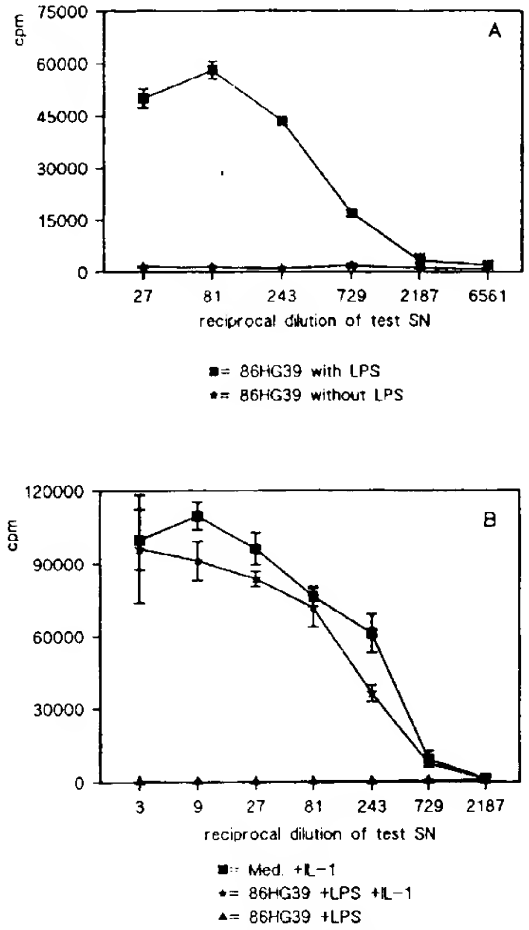

Fig. 2. A. 2×10^5 86HG39 cells were treated with 1 μg/ml LPS and supernatant was removed after 24 h and added in three-fold dilutions to 3×10^3 B9 cells (IL-6-indicator cells). B9 proliferation was measured by [^{3}H]thymidine uptake and data are given as mean cpm ± SD of triplicate cultures. B. 2×10^5 86HG39 cells were treated with 10 μg/ml LPS and the supernatant was removed after 24 h and added in three-fold dilutions to the IL-1 indicator cell EL-4. Again after 24 h, supernatant was harvested and added to 2×10^3 CTLL cells. Proliferation was measured by [^{3}H]thymidine uptake (Triangle). As positive control rhu IL-1 (30 U/ml) was added to the glioblastoma supernatant (Star) or diluted in culture medium (Square) and analysed as described above.

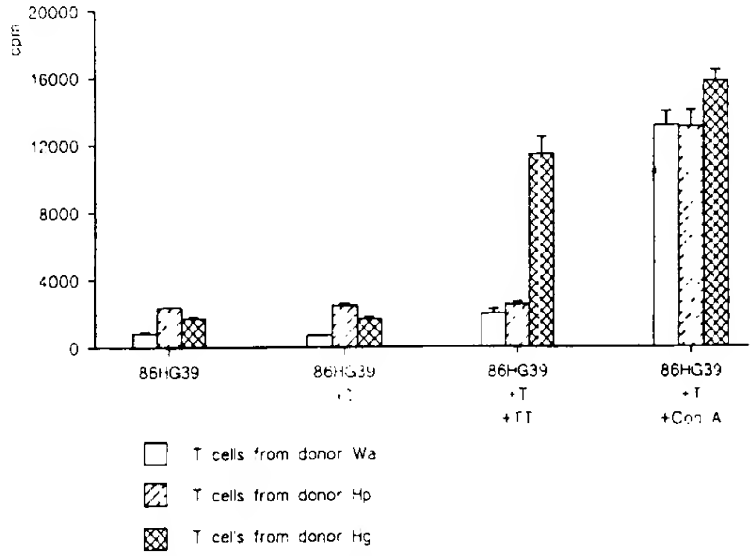

Fig. 3. 1×10^4 mitomycin-treated 86HG39 cells were co-cultured with tetanus toxoid-specific T cells from three different donors and stimulated with either tetanus toxoid (TT) or concanavalin A (ConA). T cell proliferation was monitored by [^{3}H]thymidine uptake ± SD of quadruplicate cultures.

Antigen-presenting cell function of human glioblastomas

The entire accessory cell capacity could be measured by the ability of cells to provide co-stimulatory signals in lectin-driven T cell activation. We therefore co-cultivated the glioblastoma cells together with PHA and T cell lines from five different donors. In these experiments, all three glioblastomas tested were able to induce T cell proliferation. Similar data were obtained with ConA. Representative data obtained with 86HG39 cells are shown in Fig. 3. In contrast to the results obtained by lectin stimulation, co-cultivation of glioblastomas and T cells with the mitogenic mAb OKT3 did not result in T cell activation. Carried out as a control experiment, PBMC used as accessory cells in parallel induced a strong mAb OKT3-driven T cell activation (data not shown).

In the next series of experiments, the three glioblastoma cells with and without IFNγ treatment were tested for their capacity to induce TT-specific T cell proliferation within cell lines obtained from five different donors. 87HG28 and 87HG31 cells were not able to induce TT-driven T cell proliferation within the different cell lines, even after IFNγ treatment (data not shown). In contrast, 86HG39 cells were able to induce a TT-specific T cell response when co-cultured with cells from donor Hg, while no activation of T cells from donor Wa, Vo, Th and Hp occurred. Data of a typical experiment with 86HG39 cells are shown in Fig. 3. The TT-driven activation of T cells from donor Hg by 86HG39 cells is dependent on the amount of TT and the number of 86HG39 cells used as APC (Fig. 4). In order to prove this T cell response to be really due to specific antigen presentation by 86HG39 cells and not to contaminating accessory cells within the T cell line, 86HG39 cells were treated with 3 μg/ml TT for 24 h. These TT-pulsed cells were effective in the stimulation of T cells from donor Hg, while untreated 86HG39 cells used as control were not. The addition of TT to both cell types after the addition of T cells resulted in a comparable T cell stimulation (Fig. 5).

To prove whether the capacity of 86HG39 cells to activate T cells in an antigen-specific fashion is restricted to TT-specific T cells from donor Hg, several TLA-specific T cell lines from this donor were established. The co-cultivation of these cell lines with 86HG39 cells and TLA also resulted in a strong T cell activation (Fig. 6).

Next, we investigated whether this response is MHC antigen-dependent. FACS analyses of TT- and TLA-specific T cell lines from donor Hg revealed that these cells were $CD2^+$, $CD3^+$, $CD4^+$ and $CD8^-$ and were

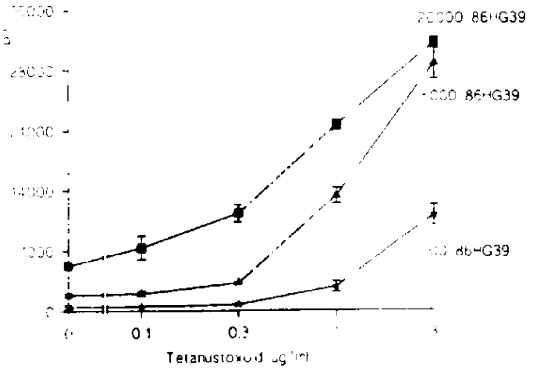

Fig. 4. Tetanus toxoid-specific T cells from donor Hg were co-cultured with different amounts of mitomycin-treated 86HG39 cells, and tetanus toxoid (0.1–3 μg/ml) was added. T cell proliferation was monitored by [^{3}H]thymidine uptake and data are given as mean cpm ± SD of triplicate cultures.

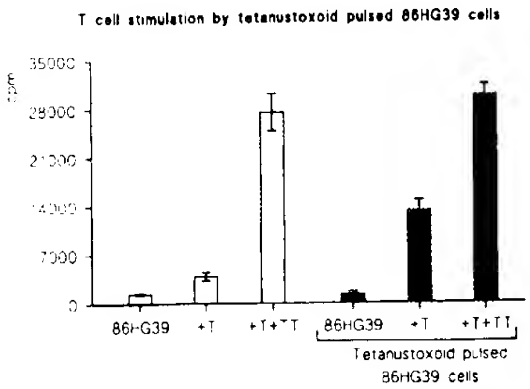

Fig. 5. 2×10^4 86HG39 cells (open columns) or tetanus toxoid-pretreated 86HG39. cells (closed columns) were added to tetanus toxoid-specific T cells from donor Hg with or without tetanus toxoid (TT). T cell proliferation is given as mean [^{3}H]thymidine uptake ± SD of quadruplicate cultures.

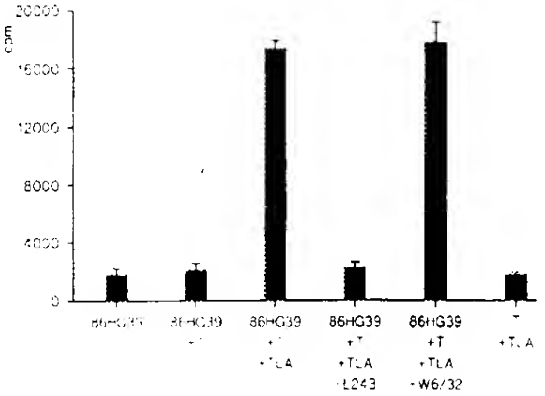

Fig. 6. 1×10^5 mitomycin-treated 86HG39 cells were co-cultured with Toxoplasma lysate antigen-specific T cells from donor Hg (T) and TLA. mAbs L243 and W6/32 were added as 1:500 diluted ascites. T cell proliferation is shown as mean cpm [^{3}H]thymidine uptake ± SD of triplicate cultures.

therefore class II antigen-restricted. mAb L243, known to inhibit HLA-DR-dependent T cell proliferation, was added to 86HG39 cells co-cultured with TLA and TLA-specific T cells. In this case, T cell proliferation was blocked (Fig. 6). Control mAb W6/32, directed against HLA class I antigens, did not alter T cell proliferation.

In order to clarify whether this inhibition was caused by interaction of antibody with 86HG39 cells rather than with the activated T cells, which were also class II antigen-positive, we carried out experiments as shown in Fig. 7: 86HG39 cells were co-cultivated with TT-specific T cells from donor Hg and TT or PHA were added. In this experiment, mAb L243 was able to block the TT-driven T cell proliferation, while the PHA-driven proliferation, which was independent on APC class II antigen expression, was not altered. Furthermore, the IL-2-driven proliferation of the T cells from donor Hg was not influenced by mAb L243, indicating that the mAb had no suppressive effect on the class II antigen-positive T cells (Fig. 7).

These data led to the conclusion that 86HG39 cells

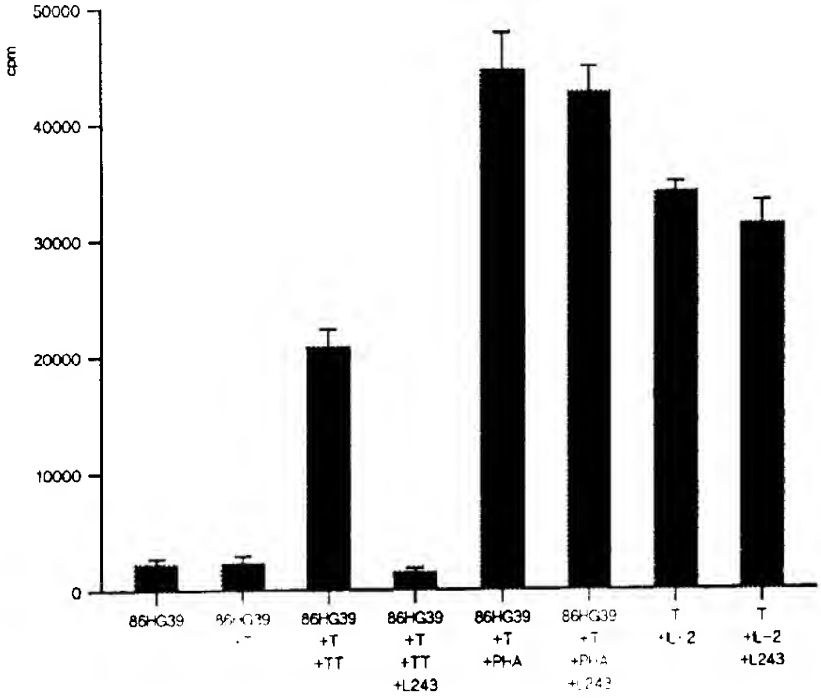

Fig. 7. 1×10^4 mitomycin-treated 86HG39 cells were co-cultured with 0.5×10^5 tetanus toxoid specific T cells from donor Hg (T), and tetanus toxoid (TT) or phytohaemagglutinin (PHA) were added. The mAb L243 was used as 1:500 diluted ascites. T cell proliferation was monitored by [^{3}H]thymidine uptake and data are given as mean cpm ± SD of triplicate cultures.

share at least one class II antigen with cells from donor Hg. The determination of HLA class II phenotype of 86HG39 cells and cells from donor A showed similarity within the HLA DQ region: both cell types were positive for $DQ_{w(3)}$. Of the HLA-DR antigens, DRw52 was found on both cell types. DRw11 expressed on 86HG39 cells and DRw13 expressed on cells of donor Hg shared eight common amino acids within the antigen-presenting region.

Discussion

T cell recognition of antigen on antigen-presenting cells requires expression of HLA molecules in their function as restriction elements. The induction of T cell proliferation is furthermore dependent on cell–cell signalling by cytokines like IL-1, IL-6, TNF and IFNγ (Vink et al., 1990). In this study, human T cell lines directed against TT or TLA were used and all cells were CD4-positive. The restriction elements for CD4-positive T cells are the HLA-class II antigens on the accessory cells.

Glioblastoma 86HG39 was derived from a recurrent glioblastoma and was found to be positive for GFAP, suggesting an astrocytic origin of this cell line. The consistent class II antigen expression on 86HG39 cells found is not like most human adult astrocytes, which are class II antigen-negative. For normal astrocytes, IFNγ treatment is necessary for class II antigen in vitro expression, or in vivo astrocytes become HLA-DR-positive at sites of inflammation. Most of the established glioblastoma cells showed the same behavior of HLA-DR expression as normal astrocytes. The glioblastoma cells 87HG28 and 87HG31 used by us were class II antigen-negative, and IFNγ was able to induce a remarkable HLA-DR antigen expression. However, the consistent HLA-DR expression by 86HG39 cells is not unique, because a few other astrocytomas were also described to be HLA-DR-positive (Carrel et al., 1982).

While antigen recognition by T cells is sufficient for the initiation of the cytolytic effector function, the induction of T cell proliferation is dependent on additional signals, for example cytokine release. IL-6 and IL-1 secretion by glioblastoma cells, two main monokines involved in T cell activation, was determined. The data presented here show that 86HG39 cells, contrary to 87HG28 and 87HG31 cells, did not produce IL-6 spontaneously, while LPS treatment of these cells resulted in a strong induction of IL-6 secretion. On the other hand, even high doses of LPS (up to 10 μg/ml) did not induce IL-1 secretion in any of the glioblastoma cell lines tested. Additionally, these cells were also found to be negative for membrane-bound IL-1, assayed by co-cultivation of the IL-1 indicator cell directly with LPS-treated glioblastoma cells. The lack of IL-1 detection within the EL-4/CTLL bioassay was not due to a suppressor factor produced by 86HG39 cells, since IL-1 was detected in the glioblastoma supernatant as well as in culture medium. Our lymphokine tests were performed in culture medium containing 5% FCS. The effect of a glioblastoma-derived suppressor factor/TGFβ2 in contrast had been observed under serum-free conditions (Siepl et al., 1988). Those different culture conditions may explain the absence of negative signals in our glioblastoma supernatants. However, this finding is in agreement with a previous study of glioblastoma cell line T24 which also failed to produce IL-1 after LPS treatment (Lee et al., 1989).

The capacity of glioblastoma cells to induce T cell proliferation was analysed in different steps. In the first set of experiments we found that the glioblastoma cell lines 86HG39, 37HG28 and 87HG31 were able to induce PHA- and ConA-driven T cell proliferation. In these experiments, T cell lines from at least five different donors were stimulated comparatively. Interestingly, only cell lines from one single donor could be activated by foreign antigen presented by 86HG39 cells. Similar data were obtained with TT and TLA-specific T cells from this donor. This antigen-specific response was shown to be dependent on the number of glioblastoma cells present in the experiment, as well as on the amount of antigen added. Furthermore, we showed that pulse treatment of 86HG39 cells with antigen was sufficient for T cell activation, indicating that the antigen was actually presented by the glioblastoma. The antigen-specific T cell response, as shown by the inhibitory effect of mAb L243, was dependent on HLA-DR antigen expression by glioblastoma 86HG39.

TT is a relatively large antigen with a M_r value of 160 000, and therefore it must be processed before it can be presented to T cells associated with HLA-DR molecules. The human GFAP-positive glioblastoma cell lines T67 and T70 were recently shown to be able to present soluble antigen on their surface, a process which is blocked by chloroquine which inhibits antigen processing by increasing the pH within lysosomes (Cussimano et al., 1990). Our data suggest that 86HG39 cells are also able to process antigen, but it cannot be excluded that small amounts of small peptides contaminating the TT preparation, or which were generated from the intact antigen within the culture time by degradation processes, associated directly with HLA-DR molecules. In the case that no processing occurs, the same mechanism has to be assumed in the activation of toxoplasma lysate antigen-specific T cells by TLA.

Analysing the HLA-class II phenotype of 86HG39 cells and cells from donor Hg, the following homologies were found. Within the HLA DQ region, DQ_{w3} is

similarly expressed on both cell types. This homology is not responsible for the described T cell activation, because mAb L243 directed against HLA-DR antigens completely blocks T cell proliferation. Within HLA-DR there are two identical restriction elements, namely DRw52 and eight amino acids within the antigen-presenting region shared by DRw11 and DRw13. The fact that, despite differences in HLA phenotype, a HLA-dependent foreign antigen-specific interaction between these cells takes place is most likely explained by the reaction of only certain subclones of T cells within the cell lines of donor Hg which were restricted to HLA-DR antigens or regions of such molecules shared by both cell types. This point of view is further supported by the finding that T cells from donor Hg, which were maintained after primary stimulation with only 86HG39 cells, TT and IL-2 for more than 6 months retained the ability to interact with 86HG39 cells as well as with EBV-transformed B cells from the same donor. On the other hand, one of the T cell lines from donor Hg frequently restimulated with syngeneic EBV-transformed B cells for more than 7 months had lost its ability to interact with 86HG39 cells. This lack of reactivity is explained by selection processes taking place within T cell lines by long-term cultures, resulting in the loss of some subclones. At the moment we are trying to find out whether frequent restimulation of T cells on accessory cells which share some or only one common HLA-DR antigen might be useful to obtain T cells restricted to different HLA molecules but specific for the same foreign antigen.

In summary, this report describes a foreign antigen-specific interaction between human brain tumor cells and T cells and hereby it is proposed that this interaction might be used as a model system to study cellular immune reactions within the CNS

Acknowledgements

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Glioblastoma-Cell-Derived T-Cell Suppressor Factor (G-TsF)

Sequence Analysis and Biologic Mechanism of G-TsF

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Vienna, Austria

Patients with glioblastoma show impaired cell-mediated immunity as manifested by cutaneous anergy to a variety of antigens and a decrease in T-cell blastogenic responsiveness *in vitro*.[1-3] In addition, T cells infiltrating glioblastoma tissue are unresponsive to mitogenic stimulation.[4] In tumor cyst fluid of patients with glioblastoma[5] and in the patients' serum, nondialyzable factors that inhibit lymphocyte proliferation can be detected before but not after tumor removal.[1]

Recently, we demonstrated that human glioblastoma cell line 308 releases a factor into the culture medium—termed "glioblastoma-derived T-cell suppressor factor" (G-TsF)—that inhibits T-cell proliferation *in vitro*.[6] Purified to homogeneity the factor was identified as a protein with a molecular weight of 12.5 kd. Aminoterminal sequence

TABLE 1. N-Terminal 25 Amino Acids of Human G-TsF[a]

	1									10										20				
h.G-TsF	A	L	D	A	A	Y	C	F	R	N	V	Q	D	N	C	C	L	R	P	L	Y	I	D	F	K
b.CIF-B	A	L	D	A	A	Y	C	F	R	N	V	Q	D	N	C	C	L	R	P	L	Y	I	D	F	K
h.TGFβ2	A	L	D	A	A	Y	C	F	R	N	V	Q	D	N	C	C	L	R	P	L	Y	I	D	F	K
h.TGFβ1	A	L	D	T	N	Y	C	F	S	S	T	E	K	N	C	C	V	R	Q	L	Y	I	D	F	R

[a] The data of the N-terminal amino acid sequence of human G-TsF are compared to the sequences of bovine CIF-B[9], human $TGF_{\beta 1}$,[8] and $TGF_{\beta 2}$.[10]

TABLE 2. G-TsF-Mediated Inhibition of Antigen and IL-2-Induced Growth of T Cells

Cultures	Additions: G-TsF	Additions: Control	OVA-7 T-Cell Proliferation (cpm ± SD)
Experiment I[a]			
OVA-7 T	−	−	705 ± 11.4
+ OVA	−	−	599 ± 9.1
+ APC	−	+	72,260 ± 4,905.2
+ APC	+	−	25,827 ± 1,314.9
Experiment II[b]			
OVA-7 T	−	−	279 ± 38
OVA-7 T + IL-2	−	+	215,015 ± 7,298
OVA-7 T + IL-2	+	−	16,831 ± 1,571

[a]*Experiment I.* OVA-7 T cells (H-2[b], 2 × 10^4) were cultured in 200 μl of serum-free Iscove's medium complete (Behring), supplemented with 2-mercaptoethanol (0.05 mM) and l-glutamine (0.3 mg/ml), for 72 hours in the presence of antigen (ovalbumin [OVA], 2.3 μM) and irradiated antigen-presenting cells (APC: 1 × 10^6 thymocytes, H-2[b]). Purified G-TsF was added at a concentration of 4 × 10^{-11}M; the G-TsF control consisted of 0.1% TFA in 2-propanol (25% v/v) used for elution of G-TsF from the final Pro-RPC column.

[b]*Experiment II.* OVA-7 T cells (1 × 10^4) were cultured as just described in the presence of IL-2 (50 U/ml) with purified G-TsF at a concentration of 1.6 × 10^{-11}M.

Fourteen hours before harvest, 1 μCi ^{3}H-thymidine per well was added.

analysis of G-TsF[7] demonstrated that 15 of 25 amino acids are identical to human transforming growth factor-β (TGFβ).[8] Identical sequences have been obtained independently for bone-derived bovine cartilage-inducing factor B (CIF-B)[9] and TG-β2 being purified from human platelets[10] (TABLE 1). When tested at a concentration of 4 × 10^{-11}M on a T-helper cell line (OVA-7 T), purified G-TsF inhibited the growth of OVA-7 T cells being activated with antigen and in the presence of thymocytes as antigen-presenting cells (TABLE 2, Experiment I). In addition, G-TsF was found to directly interfere with the growth-promoting effect of interleukin-2 on OVA-7 T cells (TABLE 2, Experiment II).

If released *in vivo,* G-TsF may contribute to impaired immunosurveillance and to the cellular immunodeficiency state detected in patients with glioblastoma.

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Malignant Glioma-Derived Soluble Factors Regulate Proliferation of Normal Adult Human Astrocytes

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Abstract. Malignant gliomas are characteristically surrounded by marked gliosis. To assess whether glioma-derived products contribute to the proliferation of astrocytes, a feature of the gliosis response, we evaluated the influence of culture supernatants from malignant human glioma lines and tumor cyst fluids collected from two patients with glioblastoma multiforme on the proliferation of non-transformed adult human astrocytes. Both the culture supernatants and cyst fluids significantly increased DNA synthesis in astrocytes as assessed by a double immunofluorescence glial fibrillary acidic protein-bromodeoxyuridine technique. The net proliferative effect mediated by glioma cell line supernatants was tumor growth phase-dependent, being preferentially expressed during the logarithmic phase of glioma cell growth. Specific growth factor molecules and cytokines known to be secreted by gliomas (epidermal growth factor, fibroblast growth factor, platelet-derived growth factor, transforming growth factor-β, interleukin-6, and tumor necrosis factor-α) could not reproduce the mitogenic effects of the glioma-derived soluble factors. Cytokines which can induce DNA synthesis by adult human astrocytes *in vitro*, gamma-interferon and interleukin-1, were not detected in the culture supernatant of glioma lines used in this study. In conjunction with the documented effects of glioma products on endothelial and lymphoid cells, the current study suggests that soluble glioma products can contribute to the production of surrounding gliosis observed *in vivo*.

Key Words: Astrocyte; Glioma; Gliosis; Proliferation; Soluble factors.

INTRODUCTION

Reactive gliosis, where astrocytes undergo hypertrophy and proliferation, is a pathologic feature of tissue that surrounds malignant brain tumors (1–4). In many cases the glial reaction forms a pseudocapsule that facilitates surgical removal of the tumor; in other instances, the periphery of the neoplasm is difficult to distinguish from reactive glial proliferation (3, 4). The cause of the reactive gliosis has been attributed to the "irritative" effect of an invasive tumor on the surrounding brain tissue. However, since gliomas themselves can secrete a range of soluble molecules (5–16), the potential role of these products also needs to be considered. Among growth factor molecules known to be produced by gliomas, epidermal growth factor (EGF), acidic or basic fibroblast growth factor (aFGF and bFGF, respectively) and platelet-derived growth factor (PDGF) have been shown to promote proliferation of neonatal rodent or fetal human astrocytes *in vitro* (17–22).

Although patients with malignant gliomas exhibit a profound decrease in systemic immunity (10, 23–26), glioma cells *in vitro* have the capability to synthesize cytokines such as transforming growth factor-β (TGF-β), tumor necrosis factor (TNF), interleukin (IL)-1 and IL-6 (10, 11, 14–16). These cytokines, if locally released by gliomas, may play a role in mediating the reactive gliosis observed around brain tumors *in vivo*, especially in view of reports that IL-1 (27–29), IL-6 (30) and TNF-α (30, 31) are mitogens for astrocytes *in vitro*. We have recently reported that gamma-interferon (γ-IFN) induces proliferation of cultured non-malignant adult human astrocytes (32).

The present study was undertaken to examine the overall effect of soluble molecules secreted by malignant gliomas on the proliferative rate of non-malignant astrocytes, one feature of the reactive gliosis response. It is noted that the process of gliosis can involve many types of changes to the astrocyte, including increases in immunoreactivity and/or content of glial fibrillary acidic protein (GFAP; the astrocyte intermediate filament), hypertrophy, proliferation and other metabolic alterations. Indeed, we have recently suggested that the mediators for many of these changes, which are not necessarily correlated, may be different (33). In the present study, we have chosen to examine one aspect of the reactive gliosis response, that of proliferation. Cultured adult human astrocytes served as proliferation targets for cell-conditioned supernatants of glioma cell lines, as well as for tumor cyst fluids extracted from two patients with glioblastoma multiforme. For the glioma cell line-conditioned media, these were collected at different stages of the glioma growth *in vitro* in order to examine whether the secretion of soluble astrocyte mitogen(s) was growth phase-specific. Further experiments involved assessing whether the astrocyte mitogenic effect could be accounted for by known glioma secretory products (EGF, FGF,

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PDGF, TGF-β, TNF-α and IL-6) or by mitogens described for adult human astrocytes (γ-IFN and IL-1) (32).

MATERIALS AND METHODS

Human Glioma Cell Lines

Glioma cell lines A172 and U563-MG are well-characterized lines established elsewhere (34, 35). These lines are passaged regularly (consisting of gentle trypsinization at 0.05% for 10 minutes followed by serum inactivation of trypsin and then repeated washes with phosphate buffered saline [PBS] and replating) and are maintained in 25 cm^2 tissue culture flasks in medium consisting of Eagle's minimum essential medium supplemented with 10% fetal bovine serum (FBS), gentamicin (20 μg/ml), glutamine (2 mM), pyruvate (1 mM), dextrose (0.1%) and essential amino acids buffered to a pH of 7.0. Cells are grown at 37°C in a humidified 5% CO_2 incubator.

Collection of Glioma Supernatants

Glioma cell lines were seeded at a density of 1×10^5 cells in 5 ml of tissue culture medium per 25 cm^2 culture flask and permitted to grow to confluency. Cell-conditioned medium (supernatant) was replaced every 2 days to avoid nutrient depletion or accumulation of metabolites that might influence the bioassays described below. For the purposes of this study, three time points for each culture were chosen to represent different stages in the growth phase of each tumor: early logarithmic growth phase, late logarithmic growth phase, and immediately following the attainment of confluency. Since the U563-MG tumor grows more slowly than the A172 (36), supernatant from days 4–6 post-seeding of U563-MG represented the early logarithmic growth phase in contrast to A172 glioma where this phase was achieved between days 2–4 post-plating. Similarly, the two later time points were obtained at intervals of days *in vitro* 4–6 and 12–14 for A172; for U563-MG, corresponding intervals were days *in vitro* 8–10 and 23–25, respectively. Supernatants were centrifuged to eliminate cellular debris and fast frozen to −70°C for storage prior to use.

Collection of Glioma Cyst Fluids and Control Cerebrospinal Fluids

Cyst fluids were collected from two patients with glioblastoma multiforme proven histologically; the fluids were aspirated to achieve decompression. Repeated aspiration through an Ommaya reservoir system was done in one patient three times at intervals of approximately 2 weeks. Cyst fluid was aspirated at time of surgical excision in the second patient. A third set of cyst fluid was collected from a patient with hemangioblastoma, a non-glial tumor that is usually of vascular origin.

As controls for the cyst fluids, cerebrospinal fluid was obtained by lumbar puncture from two subjects being investigated for herniated lumbar discs.

Astrocyte Cell Cultures

Our technique of isolation of non-malignant astrocytes has been published previously (37,38). Non-malignant human brain tissue was obtained from young adults undergoing surgical resection to ameliorate intractable epilepsy. Tissue adjacent to the epileptogenic focus was removed by Cavitron ultrasonic aspiration. For cell isolation, meninges and visible blood vessels were removed and brain tissue was cut into cubes of 1 mm or less. Viable dissociated cells were then obtained by previously established protocol using trypsin digestion and Percoll centrifugation (37, 38). Cells were suspended in feeding medium and placed in 25 cm^2 Falcon flasks for 24 hours (h) after which the floating cells (mostly oligodendrocytes) were removed for other studies. Adherent cells (mostly astrocytes and microglia) were left undisturbed and allowed to differentiate for a period of 7 days. By immunohistochemical identification, the majority of the cells were either astrocytes as assessed by GFAP immunofluorescence or presumed microglia/macrophage cells (leu-M5-positive). We have been unable to enrich for astrocytes beyond 70% purity; however, we have previously demonstrated that the mitogenic effect of γ-IFN on adult human astrocytes did not depend on the relative amounts of microglial cells that were present in culture (32). As will be indicated in the Results section, the amount of microglial cells in culture did not appear to be a factor for cyst fluids from glioma patients to elicit a proliferative response on astrocytes.

Feeding medium was Eagle's minimum essential medium supplemented with 5% FBS, 20 μg/ml gentamicin, and 0.1% dextrose. The cultures used in this study ranged in age from 2 to 4 weeks post-dissociation.

Astrocyte Proliferation Assays

GFAP-Bromodeoxyuridine (BrdU) double labeling technique: Since the non-malignant cultures contained microglial cells in addition to astrocytes, ^{3}H-thymidine incorporation would not have yielded information as to the cell type that has incorporated the proliferation label. For this reason, a double immunofluorescence technique that allows direct visualization of the cell type that has incorporated the proliferation label (BrdU) was used (17, 39). The astrocyte cultures described above were removed from their flasks by 0.05% trypsin and seeded on poly-l-lysine-coated 9 mm Aclar plastic coverslips at a density of 10^4 cells per coverslip. Cells were incubated with test supernatants (1:1 concentration with feeding medium) for 4 days, and 10 μM BrdU was added during the last 48 h to allow proliferating cells to incorporate this label. Preliminary experiments had indicated that this time frame of treatment was optimal for assessment of proliferation of adult human astrocytes. The cells were then immunostained using antibodies to GFAP and BrdU by a method that has been described in detail elsewhere (17, 39); in a recent report (33) we have shown that this method yielded results reflective of changes in actual cell numbers. In cultures treated with cyst fluids or human cerebrospinal fluids, these fluids were used at 20% final concentration (v/v) in feeding medium. All immunolabeled samples were coded and subsequently counted blindly to obtain the percentage of GFAP-positive cells that have incorporated BrdU. In cases where results were pooled from multiple series of human astrocyte cultures (Fig. 1), each with slightly varying control rates of proliferation, results were expressed as the proliferation index (PI). The PI was the percentage of GFAP and BrdU double-positive cells (proliferating astrocytes) in test cultures divided by similar results from untreated controls in the same experiment.

To attempt to account for the identity of the astrocyte mitogen in glioma-derived supernatants or cyst fluids, the following were added for 4 days to adult human test cultures for GFAP and

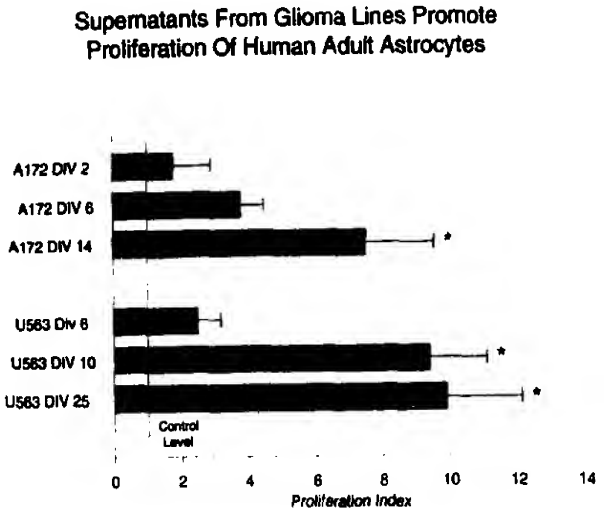

Fig. 1. Glioma supernatants promote proliferation of adult human astrocytes. Proliferation indices are mean ± SEM of four samples following a 4 day treatment period. Supernatants were collected from glioma lines A172 and U563-MG at the days *in vitro* (DIV) indicated following replating of cells (time 0). The percentage of astrocytes in control cultures that had incorporated BrdU was 2.8 ± 1.1%. ★Significantly different from control cultures using a one-way analysis of variance (ANOVA) with Duncan's multiple comparison, $p < 0.05$. All analyses were performed blindly on coded specimens.

BrdU immunofluorescence: EGF (Boehringer Mannheim, Germany; 20 ng/ml), acidic or basic human FGF (UBI, Waltham, MA; 20 ng/ml), human PDGF (Genzyme, Boston, MA; 200 mU/ml), human TGF-β (Calbiochem, La Jolla, CA; 20 ng/ml), recombinant human IL-1, IL-6 and TNF-α (all from Genzyme, Boston, MA; 100 U/ml). These were optimal concentrations that were selected based on previous studies to generate dose-response curves for these agents on neonatal mouse, fetal human or adult human astrocytes (17, 32, unpublished observations). In addition, a mixture of EGF, aFGF, bFGF (all at 20 ng/ml) and PDGF (at 200 mU/ml) was also assessed.

All experiments were conducted in feeding medium where the final concentration of FBS was 5%.

Flow Cytometric Cell Cycle Analysis: To confirm the results of the GFAP-BrdU incorporation assay, cell cycle analysis was performed by propidium iodide staining with analysis by flow cytometry. As above, the cultures were maintained for a period of 4 days with the glioma supernatants (1:1 concentration with feeding medium), after which the cells were trypsinized, washed three times in PBS, and fixed by resuspending 1×10^6 cells per ml in a solution of PBS:ice cold methanol (1:2 final concentration, vortexing the cells to avoid clumping) for a period of 1 h at 4°C. The propidium iodide staining protocol was adapted from that of Hurley (40). After fixation, cells were centrifuged and the supernatant decanted. Immunocytochemical GFAP staining was performed in 1.8 ml Ependorf tubes using 30 μl of rabbit anti-GFAP primary antibody (volume to achieve saturation of antigen) followed by equivalent volumes of goat anti-rabbit secondary antibody conjugated with fluorescein isothiocyanate (FITC), each for an incubation period of 1 h at 4°C. Each stain was followed by three washes with PBS. Following GFAP labeling, 0.5 ml propidium iodide stain solution was added. This stain solution contained 10 mg propidium iodide (Calbiochem), 0.1 ml Triton X-100 (Sigma, St. Louis, MO), and 3.7 mg EDTA (Sigma) in 100 ml PBS. Immediately following addition of the stain solution, 0.5 ml ribonuclease (RNase) solution (10 mg RNase [Sigma] mixed with 5 ml PBS and heated to 75°C for 30 minutes before use) was added to eliminate double-stranded RNA which would interfere with DNA quantification. The samples were then analyzed by flow cytometry (FACScan™, Becton Dickinson, San Jose, CA) after a 1 h staining period at 37°C in the dark. To specifically determine the proliferation of astrocytes in the mixed population, the cytometer was gated to analyze the DNA content of cells labeled with the GFAP-specific marker. Cellular DNA events were acquired using CELLFIT software (Becton Dickinson), with estimation of percentage of cells in particular phases performed by the RFIT model. Proliferation indices for flow cytometry, defined by the sum %S + %G_2/M phases, were calculated for each of the various treatments.

Measurements of γ-IFN and IL-1 in Glioma Culture Supernatants

Culture supernatants of A172 glioma cells from days 7–9 post-seeding were collected. Twenty μl, in triplicates, were analyzed for γ-IFN using a radioimmunoassay kit (Centrecore, Malvern, PA) following the manufacturer's instructions. As a positive control for the assay, 20 μl of culture medium of human CD8$^+$ T-lymphocytes activated by OKT3 and IL-2 (32) were used.

Interleukin-1 was measured in A172 culture supernatant (7–9 days post-seeding) by its ability to stimulate thymocyte proliferation in the presence of phytohemagglutinin, following published protocols (41). As a positive control for the IL-1 bioassay, 20 μl of culture supernatant collected from a highly enriched (over 95%) adult human microglia culture and treated for 24 h with 5 μg/ml bacterial lipopolysaccharide were used.

RESULTS

Effects of Glioma Cell Line Supernatants on Astrocyte Proliferation

GFAP-BrdU Double Labeling Proliferation Assay: Figure 1 shows the relative PI obtained following the incubation of glial cultures for a period of 4 days in the presence of glioma supernatants from various growth phases. Supernatants from both the A172 and U563-MG glioma lines increased the PI of the human astrocyte cultures.

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Fig. 2. GFAP-BrdU double immunolabeling technique. Adult human glial cultures in control feeding medium (upper) and following the addition of glioma supernatant (lower). Cultures exposed to the glioma supernatants show an increased number of BrdU-staining GFAP-positive cells (arrows). A, C: GFAP. B, D: BrdU.

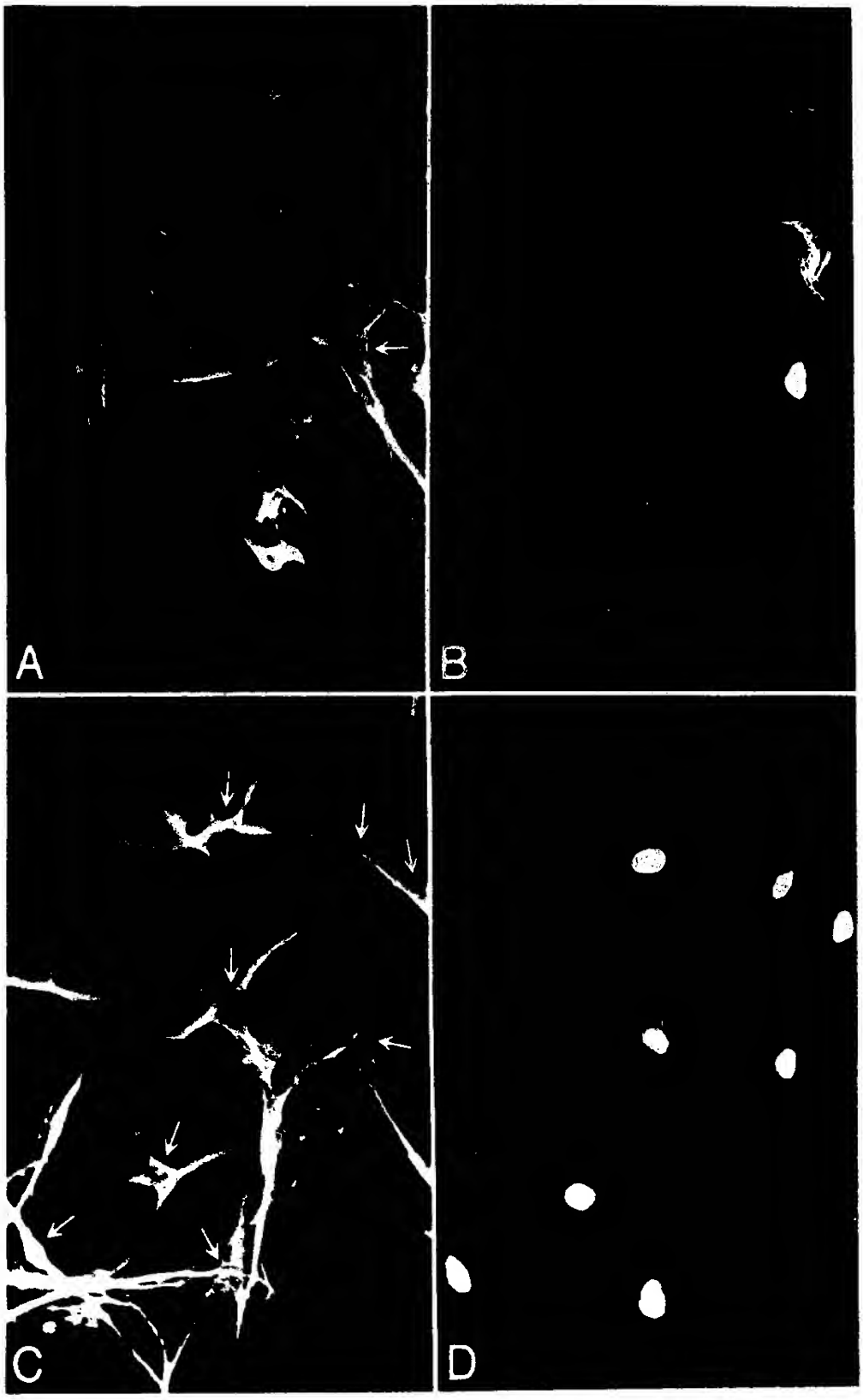
A
B
C
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TABLE 1
Flow Cytometric Analysis of Cell Cycle Kinetics of Astrocytes Following Addition of Glioma Supernatants

Treatment	%DNA synthesis (S-phase)	Proliferation index (%S + %G_2/M)
Control	0.7	6.8
A172 DIV 3	3.9	9.2
A172 DIV 11	10.2	16.2
PDB 100 nM	12.7	19.9

Values of S-phase and proliferation index were determined using the RFIT model (CELLFIT software, Becton Dickinson) from DNA histograms. Incubation of cultures with glioma supernatants produced an increase in the proliferation index of astrocytes. Positive control in this assay was the phorbol ester 4-beta-phorbol-12,13-dibutyrate (PDB), an identified mitogen for adult human astrocytes (36).
DIV = days *in vitro*.

Supernatants derived from tumors in later stages of tumor growth were more effective mitogens. Supernatants from the slower growing U563-MG cell line were more effective at promoting proliferation of the cultured adult astrocytes than were those derived from the faster growing A172 glioma line. Figure 2 illustrates visual results from the GFAP and BrdU immunofluorescence technique; more double-positive cells are evident in cultures treated with the glioma supernatants.

Control astrocyte cultures not exposed to glioma products had very low basal rates of proliferation. Over a 48 h incubation period with 10 μM BrdU, 0.1–3% of astrocytes were GFAP and BrdU double-positive. This low rate is reflective of the slow turnover of adult astrocytes *in vivo* (42). In contrast, over a 48 h pulse with BrdU, 47% and 57% of fetal human and neonatal mouse astrocytes had BrdU in their nuclei, respectively (33).

Cytofluorometric DNA Analysis: To confirm the GFAP and BrdU assays, and to determine the specific cell cycle kinetics of the astrocyte cultures, PI (defined as the sum of %S + %G_2/M phases) were determined from DNA histograms (43) following incubation of the astrocyte cultures with glioma supernatants. Supernatants from the glioma line A172 increased the PI of astrocyte cultures; supernatants from cultures in later stages of growth increased the PI more effectively (Table 1). Positive assay control was the phorbol ester and astrocyte mitogen 4-beta-phorbol-12,13-dibutyrate (PDB) (36).

Effects of Glioma Cyst Fluid on Astrocyte Proliferation

Cyst fluids collected from the patient with glioblastoma multiforme at three time points all induced proliferation of human adult astrocytes (Fig. 3). Similarly, cyst fluid from the second glioma patient resulted in an astrocytic proliferative response. The magnitude of the proliferative

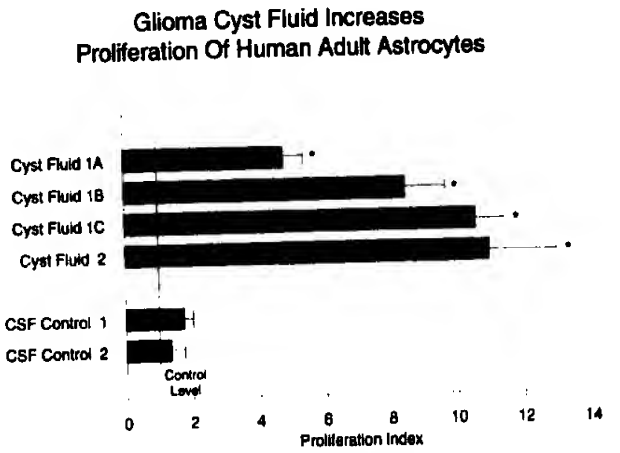

Fig. 3. Glioma cyst fluid increases proliferation of adult human astrocytes. Cyst fluid was collected from patient 1 at three time points, A, B and C at two week intervals. Cyst fluid was drained from patient 2 once. Values are mean ± SEM of four samples pooled from two adult human culture series, one with less than 20% microglial cells, and the other between 20–50% microglial cells. Control cultures (eight samples) had 3.2 ± 0.5% GFAP and BrdU double-positive cells. ★Significantly different from controls by a one-way analysis of variance (ANOVA), $p < 0.05$. Control level was proliferation index of 1 where no cyst fluid or cerebrospinal fluid (CSF) was added to sister cultures. Cerebrospinal fluid from two glioma-free and neurologically normal subjects served as additional controls.

effect (approximately tenfold) was equivalent to that produced by glioma culture supernatants at the concentrations used (compare Fig. 1 to Fig. 3). Cyst fluid from the patient with hemangioblastoma similarly enhanced proliferation of astrocytes (PI of 13.4 ± 1.1). In contrast, human cerebrospinal fluids from non-neoplastic subjects did not promote proliferation of adult human astrocytes.

Effects of Purified Growth Factors on Astrocyte Proliferation

To attempt identification of the soluble mitogen(s) in glioma cell supernatants or cyst fluids, adult human astrocyte cultures were treated with factors known to be produced by gliomas. However, none of these (EGF, aFGF, bFGF, PDGF, a mixture of these, TGF-β, IL-6 and TNF-α) enhanced proliferation of astrocytes (Table 2).

Failure to Detect γ-IFN and IL-1 in Glioma Culture Supernatants

We have previously described γ-IFN (10–1,000 U/ml) and IL-1 (0.5–500 U/ml) to enhance DNA synthesis (PI of sevenfold at best) in adult human astrocytes (32). When the A172 glioma cell culture supernatant was analyzed for the presence of γ-IFN, none was detected; the positive control used for the assay, activated $CD8^+$ T-lymphocyte supernatant, had 107 U/ml. Similarly, no IL-1 was de-

tected in the A172 culture supernatant. Adult human microglial cells treated with lipopolysaccharide for 24 h released 202 U/ml of IL-1 into the culture medium.

DISCUSSION

Malignant gliomas are known to produce a range of soluble factors which can influence their own growth. These molecules include PDGF, EGF and FGF (5–8, 12, 44), which can enhance glioma proliferation via autocrine and/or angiogenic mechanisms. The presence of insulin-like growth factor (IGF)-I and -II receptors (13) and amplification of the gene coding for the EGF receptor (5) have also been observed in higher grade gliomas. Furthermore, even though the immune status of patients with malignant gliomas is one of immunosuppression, glioma cells, at least *in vitro*, have the capacity to synthesize cytokines that include TGF-β, IL-1, IL-6 and TNF-α (10, 11, 14–16).

In addition to modulating their own growth, malignant gliomas can influence the function of non-glioma cells such as endothelial and lymphoid cells (9, 23–26, 45). In the present study, we provide evidence that glioma cells secrete soluble factors that can increase the proliferation of non-transformed astrocytes. This is seen not only in tissue culture-maintained glioma cell lines but also in tumor cyst fluids derived from two patients with glioblastoma multiforme. It is unclear why the cyst fluid from the glioma patient collected at three different time points promoted proliferation to different extents (Fig. 3); this may relate to the evolution of the glioma as is suggested by the glioma culture supernatant results (Fig. 1) where the mitogenic activity produced by glioma lines is more evident at later growth phases.

Candidate molecules for the mitogenic effect would include the glioma-produced growth factors (EGF, FGF, PDGF, IGF-I) which have been shown to promote proliferation of astrocytes (**neonatal rodent** or **fetal human**) by many laboratories including our own (17–22). However, these non-cytokine growth factors, alone or in combination, did not stimulate proliferation of **adult human** astrocytes (Table 2); we have previously shown that IGF-I was not a mitogen for adult human astrocytes (32). The failure of TNF-α in this report (in 5% FBS-containing medium) to enhance proliferation of adult human astrocytes in contrast to the report of Barna et al (31) (in 10% FBS-containing conditions) could be explained by different amounts of FBS in the test conditions; we have determined that TNF-α can be a mitogen for adult human astrocytes, but only if the culture medium contains a concentration equal to, or in excess of, 10% FBS (Tejada-Berges, Antel and Yong, manuscript in preparation).

Identified mitogens for adult human astrocytes include γ-IFN and IL-1, but these were not detected in the culture supernatant of A172 glioma cells. Heterogeneity among different glioma cell lines in the synthesis of cytokines has been documented (46), which may explain the non-detection of IL-1 in the culture supernatant of A172 while a different glioma line has been reported to synthesize IL-1 (15). Furthermore, some glioma cell lines do not synthesize cytokines *de novo* but will secrete these in response to activating agents, e.g. IL-1 (47). Thus, the identity of the astrocyte mitogen(s) released by gliomas in this study remains unknown. The current results do not rule out particular combinations of the test factors used in this study.

Interestingly, while glioma cell line-derived supernatants promoted proliferation of adult human astrocytes, we have previously reported that these supernatants suppressed lymphocyte functions at all stages of the glioma cell growth (9, 48). Furthermore, when culture supernatants from glioma cells at different stages of growth were placed on **glioma cells** (of the same cell line or on other glioma lines) in logarithmic growth phase, a biphasic response was observed: supernatants from cells in early or mid-logarithmic growth enhanced glioma growth rates while supernatants from cells at post-confluency inhibited cellular proliferation (48). These results indicate a capacity for gliomas to modulate their own growth in both a positive and negative manner, and also to affect alterations in the biologic properties of surrounding non-malignant glial cells and of tumor-infiltrating lymphocytes.

TABLE 2
Proliferative Response (BrdU Incorporation) of Human Adult Astrocytes (GFAP-positive Cells) to Test Factors

Test factor	Concentration	% of GFAP and BrdU double-positive cells
Control	—	0.8 ± 0.4 (18)
Epidermal growth factor	20 ng/ml	0.8 ± 0.5 (4)
Acidic fibroblast growth factor	20 ng/ml	0.3 ± 0.2 (4)
Basic fibroblast growth factor	20 ng/ml	0.4 ± 0.2 (4)
Platelet-derived growth factor	200 mU/ml	0 ± 0 (4)
Mixture*		0.1 ± 0.1 (4)
Transforming growth factor-β	20 ng/ml	1.9 ± 1.0 (6)
Interleukin-6	100 U/ml	2.1 ± 0.7 (4)
Tumor necrosis factor-α	100 U/ml	1.3 ± 0.1 (4)

Values are mean ± SEM with number of coverslips analyzed shown in parentheses. On each coverslip, an average of 145 GFAP-positive astrocytes was counted. * Mixture refers to a combination of epidermal growth factor, acidic and basic fibroblast growth factor and platelet-derived growth factor at the concentrations indicated. None of the experimental values are statistically significant from controls (a one-way analysis of variance [ANOVA] with Duncan's multiple comparisons). We have previously reported that biopsy-derived cultured adult human astrocytes have a low rate of proliferation, as indicated by the 0.8% value in controls above (32).

The net functional autocrine and paracrine effects of gliomas vary with the growth phase of the tumor and presumably reflect complex variations in the wide array of soluble molecules which can be produced by the glioma.

It should be noted that proliferation of astrocytes is only one aspect of the process of gliosis, which includes other alterations such as astrocyte hypertrophy, increase in GFAP immunoreactivity and/or content, and several metabolic changes such as enhancement of various mitochondrial enzyme activities. Not all of these may occur in all injury models. For example, in the facial nerve resection model, increase in the number of GFAP-immunoreactive astrocytes is observed without any documented astrocytic proliferation (49); the rise in the number of GFAP-immunoreactive astrocytes is likely due to exposure of previously hidden epitopes as the astrocyte undergoes hypertrophy. Thus, it remains to be proven that proliferation of reactive astrocytes occurs in brain regions surrounding gliomas, but the data presented here would suggest that this occurs. The data that cyst fluid from a patient with hemangioblastoma also enhances proliferation of non-malignant astrocytes *in vitro* raise the possibility that gliosis surrounding an array of non-glial neoplasms possibly including metastatic lesions may also reflect, at least in part, soluble factor effects.

In conclusion, the data from this study expand the reported effects of soluble factors derived from glioma cells on the local environment, including endothelial, lymphoid and non-malignant glial cells. We suggest that the soluble astrocyte mitogen(s) released by glioma cells can contribute to the production of surrounding gliosis in the brain.

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TI:Transforming growth factor-beta inhibits interferon-gamma secretion by lymphokine-activated killer cells stimulated with tumor cells.
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Cell therapy: achievements and perspectives

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ABSTRACT

Background and Objectives. **Cell therapy can be considered as a strategy aimed at replacing, repairing, or enhancing the biological function of a damaged tissue or system by means of autologous or allogeneic cells. There have been major advances in this field in the last few years. This has prompted the Working Group on Hematopoietic Cells to examine the current utilization of this therapy in clinical hematology.**

Evidence and Information Sources. **The method employed for preparing this review was that of informal consensus development. Members of the Working Group met three times, and the participants at these meetings examined a list of problems previously prepared by the chairman. They discussed the single points in order to reach an agreement on different opinions and eventually approved the final manuscript. Some of the authors of the present review have been working in the field of cell therapy and have contributed original papers in peer-reviewed journals. In addition, the material examined in the present review includes articles and abstracts published in journals covered by the Science Citation Index and Medline.**

State of the Art. **Lymphokine-activated killer (LAK) and tumor-infiltrating lymphocytes (TIL) have been used since the '70s mainly in end-stage patients with solid tumors, but the clinical benefits of these treatments has not been clearly documented. TIL are more specific and potent cytotoxic effectors than LAK, but only in few patients (mainly in those with solid tumors such as melanoma and glioblastoma) can their clinical use be considered potentially useful. Adoptive immunotherapy with donor lymphocyte infusions has proved to be effective, particularly in patients with chronic myeloid leukemia, in restoring a state of hematologic remission after leukemia relapse occurring following an allograft. The infusion of donor T-cells can also have a role in the treatment of patients with Epstein-Barr virus (EBV)-induced post-transplant lymphoproliferative disorders. However, in this regard, generation and infusion of donor-derived, virus specific T-cell lines or clones represents a more sophisticated and safer approach for treatment of viral complications occurring in immunocompromized patients. Whereas too few clinical trials have been performed so far to draw any firm conclusion, based on animal studies dendritic cell-based immunotherapy holds promises of exerting an effective anti-tumor activity. Despite leukemic cells not being immunogenic, induction on their surface of co-stimulatory molecules or generation of leukemic dendritic cells may induce antileukemic cytotoxic T-cell responses. Tumor cells express a variety of antigens and can be genetically manipulated to become immunogenic. The main *in vitro* and *in vivo* functional characteristics of marrow mesenchymal stem cells (MSCs) with particular emphasis on their hematopoietic regulatory role are reviewed. In addition, prerequisites for clinical applications using culture-expanded mesenchymal cells are discussed**

Perspectives. **The opportuneness of using LAK cells or activated natural killer (NK) cells in hematologic patients with low tumor burden (e.g. after stem cell transplantation) should be further explored. Moreover the role of new cytokines in enhancing the antineoplastic activity of NK cells and the infusion of selected NK in alternative to CTL for graft versus leukemia (GVL) disease (avoiding graft versus host disease (GvHD) seems very promising. Separation of GVL from GvHD through generation and infusion of leukemia-specific T-cell clones or lines is one of the most intriguing and promising fields of investigations for the future. Likewise, strategies devised to improve immune-reconstitution and restore specific anti-infectious functions through either induction of unresponsiveness to recipient alloantigens or removal of alloreactive donor T-cells might increase the applicability and success of hematopoietic stem cell transplantation. Cellular immunotherapy with DC must be standardized and several critical points, discussed in the chapter, have to be properly addressed with specific clinical studies. Stimulation of leukemic cells via CD40 receptor and transduction of tumor cells with co-stimulatory molecules and/or cytokines may be useful to prevent a tumor escaping immune surveillance. Tumor cells can be genetically modified to interact directly with dendritic cells *in vivo* or recombinant antigen can be delivered to dendritic cells using attenuated bacterial vectors for oral vaccination. MSCs represent an attractive therapeutic tool capable of playing a role in a wide range of clinical applications in the context of both cell and gene therapy strategies.**

Key words: cell therapy

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The role of lymphoid cells in rejecting solid tumors transplanted into animal models was strongly suggested in the first decades of this century by J.B. Murphy (1926)[1] who, nonetheless, did not demonstrate it formally. Following his revolutionary findings on the immunologic mechanisms of allogeneic skin tolerance and rejection, in 1958 P.B. Medawar[2] coined the term "*immunologically competent cell*" to define a cell that is "*fully qualified to undertake an immunological response*". Forty years after Medawar's definition, the development of molecular and biological research has enormously improved our understanding of the complex regulatory mechanisms of proliferation, differentiation and function of the cells involved in the immune response. The concomitant evolution of biotechnology has also progressively given new opportunities to isolate and/or expand cell subsets, or to develop new molecules, in order to amplify or modify specific cell functions. Thus, the possibility of exploiting a specific cell function, *in vivo* or *ex vivo*, to obtain a therapeutic effect, such as an anti-tumor cytotoxic activity, or complete immune reconstitution, is part of the definition of cell therapy that is herein reviewed.

In a general context, cell therapy can be considered as a strategy aimed at replacing, repairing, or enhancing the biological function of a damaged tissue or system by means of autologous or allogeneic cells. For instance, in the hematopoietic system cell therapy may include: a) removal or enrichment of various cell populations; b) expansion of hematopoietic cell subsets; c) expansion or activation of lymphocytes for immunotherapy; and d) genetic modification of lymphoid or hematopoietic cells, when these cells are intended to engraft permanently or transiently in the recipient and/or be used in the treatment of a disease.

This review contains extensive considerations on the clinical use of lymphocytes and/or natural killer (NK) cells as a strategic weapon in preventing or curing the neoplastic relapse after chemotherapy and/or hematopoietic stem cell transplantation, the infusion of T-cell clones or lines able to restore a specific antiviral activity, the *in vivo* and *ex vivo* potential use of dendritic cells to generate a tumor-specific cytotoxic activity, and the innovative use of donor stromal cells in conjunction with stem cell transplantation. Even tumor cells engineered to express cytokine or co-stimulatory molecules and representing the entire antigenic repertoire of a certain neoplasia can be used as a cancer vaccine. On the other hand, a broad definition of cell therapy at this time should include autologous and allogeneic transplants of purified hematopoietic stem cells, which, however, have been extensively reviewed in previously published reports.[3-5]

Tumor escape from immune surveillance

Although several mechanisms allowing tumor cells to escape the host immune protection have been recently described, it is conceivable that others remain still undiscovered. However, tumor cells often fail to induce specific immune responses because of their inability to function as competent antigen presenting cells (APC). Professional APC, in fact, are fully capable of delivering two signals to T cells:[6] the first is antigen (Ag) specific and is mediated by the interaction of MHC molecules carrying antigenic peptides with the T-cell receptor (TCR), and the second signal, or co-stimulatory signal, is not Ag-specific and is principally mediated by members of the B7 family, namely B7-1 (CD80) and B7-2 (CD86), via their T-cell receptors CD28 and CTLA-4, and/or by CD40 via CD40L binding.[7,8]

The lack of a suitable tumor-associated antigen (TAA),[9,10] or defective antigen processing,[11] or production of immunologic inhibitors,[12] or lack of co-stimulatory signaling by tumor cells,[13] as other mechanisms, can all contribute to prevent or abrogate an anti-tumor immune response. Moreover, neoplastic cells within the same tumor may show different reactivity with monoclonal antibodies (mAbs), cytotoxic T-lymphocyte (CTL) clones and lymphokine-activated killer (LAK) or tumor infiltrating lymphocyte (TIL) populations. Furthermore, despite many tumors having TAA and potentially being capable of stimulating T cells, in some cases they fail to induce an adequate CTL frequency *in vitro*. In other cases the antigen loss can be one of the mechanisms for escaping immune protection.[14] Private TAA often result from mutated gene products[15] and are potentially useful for developing tumor vaccines. These Ags, however, can be down-regulated or modified by point mutations, inducing a consistent reduction or the abrogation of peptide-binding by specific CTLs. Another critical issue for preventing immune responses is the absence, or the down-regulation of MHC molecules on neoplastic cells, as shown in animal models,[16] or in human lung cancer.[17]

The pivotal role of B7 molecules in the immune response has been demonstrated in a variety of experimental models showing that after TCR signaling, binding of CD28 induces T-cell interleukin-2 (IL-2) secretion, proliferation and effector function, whereas presentation of the antigen in the absence of co-stimuli induces T-cell unresponsiveness either by anergy or clonal deletion. Therefore, since most neoplastic cells lack co-stimulatory molecules, it is likely that they can deliver the first signal through the MHC:TCR binding, but not the second one, thus driving host T-cells to tolerate the tumor.[18] Potential strategies to prevent or to reverse T-cell tolerance by CD28 or CD40L stimulation, or IL-2 receptor triggering, are under investigation.

Further mechanisms impairing immunologic responses include the suppression of cytotoxic activity by the release of soluble factors or by direct cell-contact. In fact, tumor cells may secrete cytokines, such as MIP-1α, or TGF-β, or IL-10, that may be capable of inhibiting T cell activity. Alternatively,

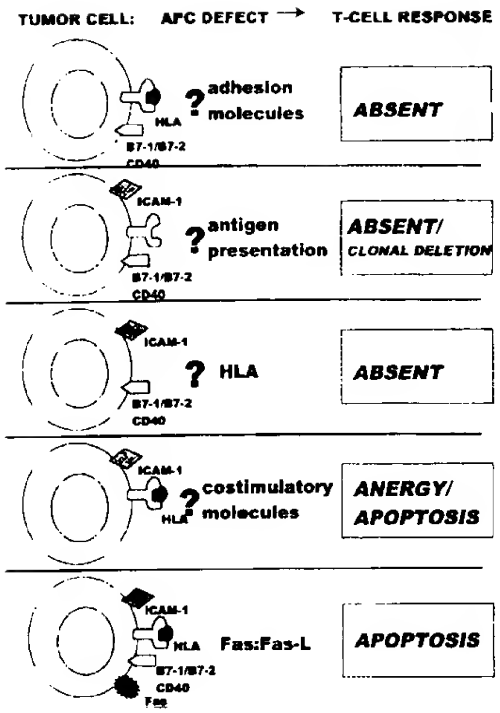


Figure 1. Main mechanisms for tumor escape of immune surveillance.

tumor cells may induce T-cell apoptotic clonal deletion by increasing Fas:Fas-L ligation.[19] A schematic example of the main defects described in the tumor cell: T cell interaction is shown in Figure 1.

Finally, since normal lymphocytes can bind to venular endothelial cells through adhesion receptors, such as L-selectin or α/β integrins, and then by rolling out they can reach tissues, lack of adhesion receptors on tumor vessels might prevent lymphocytic infiltration and contact with neoplastic cells.[20] In this case even the best strategies aimed at modifying the immunogenicity of tumor cells may not be successful at overcoming the lack of an antitumor immune surveillance.

Lymphokine-activated killer and tumor-infiltrating lymphocytes: past and present

Natural killer cells and lymphokine-activated killer phenomenon

Since 1970 NK cells have been recognized as a functionally distinct subset of cytotoxic effectors (Table 1). NK cells from rodent or from human peripheral blood kill a wide range of tumor cells and virus-transformed cells without the need for prior sensitization.

In 1975 Heberman *et al.*[21] described a phenomenon of normal unstimulated lymphoid cells lysing cultured tumor-cell lines in a short *in vitro* assay. This cytolytic activity was subsequently shown to be neither MHC restricted nor mediated by the T-cell recep-

Table 1. Characteristics of cytotoxic effectors useful for adoptive immunotherapy of cancer.

Effector type	*CTLs*	*TILs*	*NK cells*	*LAK cells*	*CIKs*
Source	Peripheral blood lymphocytes	Metastatic lymph nodes	Peripheral blood and bone marrow	NK cells and CTL activated by IL-2	Subset of T-lymphocytes activated by cytokines
Culture conditions:					
Tumor stimulation	Yes	None	None	None	None
need of IL-2 for response	++++	++++	++++ ($CD56^{dim}$) + ($CD56^{bright}$)	-	++++,IFN-γ, IL-12, anti-CD3 antibody
Duration of culture	6 weeks	4 weeks	2-3 weeks	2-5 days	2-5 weeks
target cells *in vitro*	allogeneic cells	autologous tumoral cells	K-562	Raji, Daudi	autologous and allogeneic tumoral cells
In vitro cytolytic activity:					
Specificity	MHC restricted to allogeneic cells MHC not restricted toward opsonized cells (ADCC) antigens)	Restricted to autologous tumor (MHC and/or tumor associated	none: spontaneous lysis of virus-infected cells, autologous tumoral cells, allogeneic tumoral cells antibody-dependent cell-mediated cytotoxicity (ADCC) specificity	none: lyse a wide spectrum of tumor cells including cells that are resistant to NK;	cytotoxic activity superior to LAK; lyse whether CML autologous or allogeneic blasts but do not lyse normal hematopoietic progenitors
Effector phenotype	-CD3+/4+ ,CD3+/8+ -CD3+/8+/16+	CD3+/8+/56+	CD3-/CD16+/CD56+	CD56+ CD25+	CD3+/56+

CTL: cytotoxic T-lymphocytes; TIL: tumor infiltrating lymphocytes; NK: natural killer; LAK: lymphokine activated killer; CIK: cytokine activated killer.

tor complex. Such ability to eliminate tumor cells, but not normal tissues suggests that NK cells are not only involved in the control of cancer, but also that their presence and state of activation are important in the outcome of the disease and finally in the treatment of tumors.[22]

Mature NK have a clonally-distributed ability to recognize their target cell by class I MHC alleles. Karre *et al.*[23] demonstrated in a murine model that leukemia cell lines lacking certain MHC class I molecules were killed by NK cells, while parental H-2 bearing line were not. In humans both NK and a subset of cytotoxic T-lymphocytes express receptors for MHC HLA class I molecules which exert an inhibitory effect on cell-mediated cytotoxicity. These surface molecules, belonging to the immunoglobulin superfamily, have been termed killer-cell inhibitory receptors (KIRs). Two distinct KIR families have been described a) KIRs with IG-like domains, recognizing HLA-A, B and C alleles; and b) the CD94/NKG2A subtype, with a lectin domain, recognizing peptides related to the HLA-E class I system.[24] The interaction between KIRs and the corresponding MHC class I antigens prevent NK from killing target cells expressing self HLA alleles.[25] In addition some NK also express receptors that induce lysis of target cells expressing foreign HLA class I alleles.[26]

These findings explain the mechanism of self-tolerance in the NK population, which can be disrupted as a consequence of tumor transformation or viral infection or any other events inducing a loss or a substantial modification of class I molecules. These transformed cells can easily escape detection by T-lymphocytes by down regulating MHC antigens, but are normally destroyed by autologous NK cells.[27]

The NK cell compartment is heterogeneous and distinct NK subsets have been characterized. The most informative functional differences are based on relative CD56 fluorescence: only $CD56^{+bright}$, but not $CD56^{+dim}$ NK, express the high-affinity IL-2 receptor, and respond to the low IL-2 concentration. They also expand 10 times more than $CD56^{+dim}$.[28]

NK progenitors differentiate into immature NK in presence of SCF, IL-7, IL-2 and bone marrow stromal cells producing IL-15. This last cytokine can directly induce $CD34^+$ cells to differentiate into NK cells in the absence of IL-2.[29] The second step of NK maturation is stroma-independent and is characterized by the appearance of CD56 molecules: the intensity of CD56 expression reflects the proliferative potential and the killing ability of the NK.[30]

The effects of IL-2 on NK precursors appears to be stage-specific, confirming that, while mature NK precursors readily respond to IL-2, more immature progenitors need complete mixtures of cytokines and stromal cells. NK cells, after incubation with IL-2, become lymphokine activated killer cells: LAK cells kill NK-resistant cell targets (e.g. Daudi cell line) and a wide spectrum of different fresh tumor cells in both autologous and allogeneic settings, while fresh normal tissues are resistant to LAK-mediated lysis.[31]

Although some tissue-resident lymphocytes may have spontaneous LAK activity, normal blood mononuclear cells (MNC) do not show any LAK activity, which can be acquired only after incubation with interleukin-2.[32] These NK activated cells express new markers such as CD25, MHC class II antigens and fibronectin.[33] LAK activity can be generated not only in peripheral blood MNC, but also in the thymus, spleen, bone marrow and in MNC from lymph nodes. Many experimental data suggest that most LAK precursors are present in the null lymphocyte population.

In humans LAK activity was much more evident in the MNC population after depletion of macrophages, T and B-cells. Residual MNC were $CD16^+$ and did not show T-cell markers.[34]

LAK cells: experimental observations and clinical trials

In animal models the combined administration of IL-2 and LAK has proved to be more efficacious than either component alone. In murine models the administration of high-dose IL-2 alone or in conjunction with LAK cells induced the regression of lung, liver and subdermal metastases. The antitumor effect correlated both with the IL-2 dose and the number of LAK cells administered; finally at different doses of IL-2, the concomitant administration of LAK cells resulted in increased reduction in established metastases.[35,36]

LAK cells are capable of inhibiting acute myeloid leukemia (AML) progenitor growth, and leukemia incidence is higher in people with deficiency of NK cells.[37] In the large majority of patients at diagnosis or in relapse blasts appear resistant to lysis by autologous LAK cells. Moreover, about 90% of patients with acute leukemia in complete remission do not show spontaneous cytotoxicity against autologous blast cells, but *ex vivo* treatment with IL-2 restores cytolytic activity in 37.5% of these patients.[38] In a population of 42 patients with AML in complete remission, LAK cytotoxicity against autologous leukemic blasts was not significantly different from LAK of normal subjects.[39] However, multivariate analysis for prognostic factors showed that patients whose LAK had more lytic activity on leukemic blasts had significantly less risk of relapse than patients with poor LAK activity.

In the first National Cancer Institute trial endstage cancer patients received high-dose bolus IL-2 therapy for 3 to 5 days.[35] Lymphocytes harvested during the systemic treatment with IL-2 were cultured in the presence of IL-2 for 2 to 4 days, in order to expand the LAK cell number; autologous LAK cells were then reinfused into patients in combination with the high-dose intravenous bolus IL-2 administration. Of 72 patients with renal cancer who were treated, 33% obtained an overall response, 8 with complete

response (CR) and 17 with partial response (PR); of 48 patients with metastatic melanoma 21% responded with 4 CR and 6 PR; responses were also observed in patients with colorectal carcinoma and non-Hodgkin's lymphoma.[40] The ILWG used the same strategy, obtaining an overall response rate of 19% in patients with melanoma and 16% in those with renal carcinoma.[41] After these initial trials the original schema of the National Cancer Institute was modified with the use of IL-2 in continuous infusion rather than bolus injection in order to reduce the systemic toxicity.[42]

The first randomized study, comparing IL-2 alone to IL-2 plus LAK cells, was published by McCabe.[43] This trial included patients with either renal carcinoma or melanoma; no significant difference in response rate between the two groups was reported. A second randomized study at the National Cancer Institute followed these pioneering experiences, comparing IL-2 alone to IL-2/LAK cells:[44] 181 patients were enrolled in this study (90 in the IL-2 plus LAK arm and 91 in the IL-2 alone). A total of 10 CR were observed in the IL-2/LAK arm as compared to only 3 in the IL-2 alone arm. The overall response rates were similar, but there was a survival trend (p=0.07) in favor of the IL-2/LAK arm: the actuarial survival for patients receiving IL-2/LAK was 31% compared to 17% for those receiving IL-2 alone. Toxicity was virtually equivalent in both arms and the majority of toxic effects were due to IL-2 administration, while the only complication associated with LAK therapy was transient hepatitis A, due to contamination of the culture medium.

A third randomized trial, comparing IL-2 alone versus IL-2/LAK therapy was published in 1995.[45] In this study only patients with advanced renal carcinoma were treated and IL-2 was administered as a continuous infusion rather than bolus injection. Seventy-one patients entered (36 vs. 35) this trial and only 6% overall obtained a major response, with a median survival of 13 months; the difference between the two groups was not significant. Therefore it may be concluded that LAK cells did not improve the activity of IL-2 in patients with advanced renal carcinoma.

Table 2. Clinical trials with LAK cells.

				Treatment schedule			
Author	*Year*	*Patients*	*Kind of tumor*	*IL-2(dose and schedule)*		*LAK cells*	*Response*
Rosenberg	1987	157	Melanoma	Randomize: IL-2	vs.	IL-2+LAK	CR: 2.2% vs 7.5% PR: 10.9% vs 14.2% mR: 2.2% vs 9.4%
West	1987	40	Miscellaneous	1-7x10^6 U/m^2/day CI			CR+PR: 22-28%
Yoshida	1988	23	Brain tumor	Direct injection of LAK into recurrent tumor cavity + IL-2 (50-400 U); multiple treat			Regression: 26%
Fisher	1988	29v	Renal carcinoma	12.9 MIU/kg (median 10 doses)		7x10^{10} cells	OR: 16%
West	1989	30	Renal carcinoma	3x10^6 U/m^2/day CI		NR	22-28%
Dutcher	1989	32		100,000 U/kg q8h		8.9x10^{10}	CR+PR: 19%
Paciucci	1989	24	Miscellaneous	1-5x10^6 U/m^2/day CI		5.6x10^9	CR+PR: 20.8%
Negrier	1989	51	Renal carcinoma	3x10^6 U/m^2/day CI		1.2x10^{10}	CR+PR: 27%
Stahel	1989	23	Miscellaneous	3x10^4 U/kg q8h		5.1x10^{10}	CR+PR: 17%
Rosenberg	1993	181	Metastatic cancer	Randomized: IL-2	vs	IL-2+LAK	CR: 5% vs 11.76% PR: 15.2% vs 16.5% OS (3 yrs): 17% vs 31% (p2=0.089)
Bajorin		49	Renal carcinoma	Randomized: IL-2 (3 MU/m^2)	vs	IL-2+LAK (73x10^9)	No difference
Keilholz	1994	9	Liver metastic carcinoma	IL-2 CI into the splenic artery or intravenous infusion		LAK transfer into the portal vein or the hepatic artery	CR+PR: 33%
Murray Law	1995	66	Renal carcinoma	Randomized: IL-2 (3x10^6 U/m^2/day)	vs	IL-2+LAK (NR)	CR+PR: 9% vs 3% (p=0.61)
Kimura	1997	82,788	Resected lung carcinoma	Randomized: IL-2+LAK vs (7x10^5 U/day x 3 days)		Standard therapy (1.5x10^9 cell)	OS (5 yrs): 56.1% vs 47.2% OS (9 yrs): 33.1% vs 31.2%

The last randomized trial published was conducted in 174 primary lung carcinoma patients after surgery, comparing the adjuvant treatment with IL-2 plus LAK (for two years) with conventional treatment.[46] The 5- and 9-year survival rates were significantly superior in patients receiving IL-2/LAK therapy, but no comparison was planned between Il-2 alone and IL-2/LAK therapy. The impressive results obtained in terms of overall survival also in non-curative cases after surgery (OS: 52% at 5 years) should probably be interpreted as due to fact that in this study patients received the immunotherapy after consistent tumor debulking.

Other clinical trials (non-randomized) were conducted with IL-2 with or without LAK cells, and the overall response rate was similar for both the immunotherapy modalities.[47,48] The detailed review of other (non-randomized) experiences using these two different immunotherapies suggests that LAK cell reinfusion slightly increased the number of CR and the duration of response, especially in patients with metastatic melanoma (Table 2).[49,50]

In hematologic malignancies the first attempts to generate and expand LAK activity by using IL-2 *in vivo* were clinically disappointing especially in patients autotransplanted for ALL; after transplantation patients were randomly assigned to treatment with systemic IL-2 (without LAK cell administration) or no treatment, but the disease-free survival was similar in the two arms.[51] The use of LAK cells has also been proposed after autologous transplantation for hematological malignancies, but the very small series of patients reported does not allow any definitive conclusion to be drawn about its clinical benefit.[52] Beaujean *et al.*[53] reinfused, after myeloablative therapy, BM incubated with IL-2 into 5 ALL patients, observing a very marked delay of the engraftment and the recurrence of disease in all patients. Recently there has been a report of 61 women with breast cancer autotransplanted with IL-2 activated PBPC and treated with low dose IL-2 starting from PBPC reinfusion, without graft failures or major toxicity; there are no data concerning the outcome of patients and this experience only confirms the feasibility of the approach.[54]

In a very preliminary experience a sustained major cytogenetic response to immunotherapy with GM-CSF+IL-2 and LAK infusion was observed in chronic myeloid leukemia (CML) patients after autologous transplantation.[55] However, a renewed interest in this approach has led to new research pursuing different directions:

a. selection of patients with low tumor-burden and with significant *in vitro* LAK activity against autologous tumor cells, in order to reach an optimal effector/target ratio;
b. harvest of large amounts of NK cells (for additional *ex vivo* expansion/activation with IL-2) to be reinfused in the early phase after BMT;
c. direct activation of leukapheresis, after priming with chemotherapy followed by cytokines, in order to reinfuse, after HDT, a product richer in cytotoxic effectors and probably less contaminated;[57]
d. identification and selection of more efficient NK progenitors (e.g. adherent NK) by eliminating undesired accessory cells which could inhibit their killing and proliferative ability;[58,59]
e. generation and expansion of other CE subsets with more powerful activity against autologous tumor cells, e.g. cytokine-induced killer cells (CIK);[60]
f. use of other cytokines in association with IL-2, in order to potentiate the activity and/or improve the selectivity of activated peripheral blood MNC.

Tumor infiltrating lymphocytes

The disappointing results of adoptive immunotherapy with blood-derived LAK cells led to a search for more specific CE cells. Tumor infiltrating lymphocytes (TIL) are T-lymphocytes with unique tumor activity that infiltrate some tumors and can be expanded by long-term culture with IL-2 at low-intermediate concentrations.[61] In murine models TIL have exhibited a stronger anti-tumor effect than LAK cells on a per-cell basis; in humans TIL have been isolated with variable frequency from different solid tumors and very often (about 30% of cases) from patients with melanoma. Phenotypic analysis showed that TIL consisted mainly of $CD4^+$ cells in colon, breast and urothelial tumors, while in melanoma $CD8^+$ cells are prevalent.[62,63] $CD3^-$ $CD16^+$ NK cells have also been isolated from several tumors, confirming the large heterogeneity of tumor infiltrates.[64] The mechanism of the antitumor action of TIL is unknown; there is some evidence that these cells secrete cytotoxins and cytokines which are capable of killing tumor cells and recruiting other CE

Experimental models and clinical trials

Mice carrying spontaneous metastases, treated with IL-2 plus tumor-derived T-cells, obtained from splenocytes after mixed lymphocyte-tumor cultures, had a better survival than those treated with LAK cells; previous tumor debulking (with chemotherapy and/or radiotherapy) was needed to maximize the efficacy of TIL-therapy.[65]

Unfortunately large amounts of TIL can be collected very rarely, and the large scale expansion of this population is crucial in order to obtain relevant clinical responses; this step of *ex vivo* manipulation is not always successful, because the need for prolonged culture of TIL (from 6 to 8 weeks with IL-2) may abrogate the selectivity against the tumor; moreover only a small fraction of the readministered human TIL is able to concentrate in the tumor sites.[66]

Wong *et al.*[67] showed in a mouse model that TIL preferentially localize in the liver and lungs. In contrast trafficking studies employing TIL radiolabeled

Table 3. Clinical trials with LAK cells and IL-2.

Author	Year	Patients	Kind of tumor	Treatment schedule		Response
				IL-2(dose and schedule)	TIL cells	
Rosenberg	1988	20	metastatic melanoma	1×10^5 U/kg every 8h; CPM 25 mg/kg	20.5×10^{10} cell	Regress. 60%
Kradin	1989	38	miscellaneous	1.3×10^6 U/m² CIx 24h		OR: 26%
Rosenberg	1990	5	metastatic melanoma		TIL gene modified	
Aoki	1991	10	advanced or recurrent ovarian cancer		TIL after single CI CPM	OR: 70% Long term: 57%
Dillman	1991	21v	metastatic melanoma	18×10^6 IU/m²/day CI	10^{11} cell	OR: 24% expensive, difficult
Anenti	1993	12v	metastatic melanoma	130 $<10^6$ IU/m²/day CI	6.8×10^9 cell	RR 33%
Belldegrun	1993	10v	metastatic renal cell carcinoma	$2 \cdot 10^6$ IU/m²/day in 96h (IL-2) $6 \cdot 10^6$ IU/m²/day (IFN-γ)	TIL	CR 30%
Schwartz-entruber	1994	41	melanoma	IL-2	TIL	CR+PR: 21.9%
Pockaj	1994	38	metastatic melanoma	7.2×10^5 IU/kg every 8h	$1.3\text{-}2.2 \times 10^{11}$ cell and CPM 25 mg/kg	OR 38.5%
Chang	1997	20v	advanced melanoma and renal cell cancer	IL-2	anti-CD3 vaccine primed lymph node cells activated	OR 33.3% PR 9.1%
Curti	1998		solid tumor and NHL	9×10^6 UI/m²/day x 7 days CI	T CD4+ cell+ anti CD3	some tumor regression
Ridolfi	1998	32	miscellaneous	12-6 MIU/ day (West's schedule)	5.8×10^{10} TIL	no response inpatients with advanced cancer

ev: evaluable; PR: partial response; OR: overall response.

with In^{111}, have shown that TIL do traffic to tumor sites;[68] this homing property should produce high concentrations of TIL, and probably their permanence, in the area of a tumor.

Human TIL transfected *in vitro* with the neomycin-resistance gene and reinfused intravenously, have been detected by polymerase chain reaction (PCR) techniques from 6 to 60 days in patients affected by metastatic melanoma.[69] Aebersold *et al.*[70] observed a strong correlation between the tested tumor cytotoxicity *in vitro* and the *in vivo* response, in a small cohort of patients with metastatic melanoma. A similar relationship was observed in a murine model in which the *in vivo* therapeutic effect of TIL correlated with secretion of IFNγ and tumor necrosis factor (TFNα).[71]

In order to increase their specificity and potency, TIL have been engineered with genes encoding cytokines or cytotoxins such as TNF or IFN-γ or IL-2.[69,72] However, some experimental observations suggest that these high concentrations of cytokines can cause systemic toxicity and in some cases could even make the tumor more aggressive.[73,74]

In addition to their potential therapeutic use as cytolytic effectors, the ability of some TIL to recognize unique antigens on tumor cells has made the study of the biologic characteristics of these antigens more feasible. Melanomas from different patients who share MHC antigens are often cross-recognized by allogeneic TIL, as could be expected for an MHC-restricted T-cell response; the presence of shared antigens in different patients with melanoma suggests the possibility of using these antigens in an active immunization program for this disease.[75] When adoptively transferred into patients, TIL showed significant therapeutic efficacy in patients with advanced melanoma, but not in renal carcinoma patients. In a phase II trial patients with malignant melanoma were treated with IL-2 and TIL following chemotherapy:[76] 39% of them achieved some sort of response, including those who had previously experienced a failure of IL-2 therapy. Kradin *et al.*[77] treated some patients with a combination of chemotherapy, IL-2 and TIL, obtaining 23% of responses in those affected by melanoma and 29% in those with renal carcinoma, but none in patients with non-small cell lung carcinoma.

A summary of most clinically relevant clinical trials with TIL is given in Table 3.

The lack of important clinical trials with TIL is probably due to the difficulties in finding TIL at diagnosis and especially because the techniques for TIL prim-

ing and expansion are time-consuming and not completely standardized. TIL therapy is still young, but its very interesting potential has not yet been thoroughly investigated.

New approaches with LAK or TIL cells

Allogeneic setting. Whereas it is widely accepted that graft-versus-host disease (GvHD) is initiated by donor T cells recognizing foreign host antigens, other factors including toxicity of conditioning regimens and cytokine dysregulation are involved in the pathogenesis of GvHD.[78,79] Data from murine experiments show that NK cells play an active role both in GvHD and in garft-versus-leukemia (GVL) events: in a recently published model 100% of SCID mice bearing human leukemic cells, and transplanted with NK$^+$ T-cells, died of acute GvHD; but while animals which received only T-cells developed clinical GVL associated with relevant chronic GvHD, NK-transplanted animals showed the same degree of protection from leukemia, experiencing only mild-moderate acute GvHD without chronic GvHD.[80] These data suggest that in order to optimize the GVL effect while minimizing the severity of acute GvHD, donor grafts should be manipulated by adding a moderate dose of T-cells in the early phase and using purified NK cells in the late phase after transplantation.

Preliminary data suggest that in normal donors, after G-CSF mobilization, NK progenitors have decreased killing capacity and diminished proliferative ability in response to IL-2, compared to the unprimed bone marrow counterpart.[81] In contrast, after an HLA incompatible transplant, a progressive expansion of NK and CTL with NK like function (CD3$^-$/CD56$^+$) has been observed; recipients received the T-cell depleted graft without developing GvHD, but in most cases a significant GVL effect could be demonstrated both *in vitro* and *in vivo*; these data support the critical role of CTL KIR$^+$ in this particular subset of transplanted patients.[82]

Concerning the expanding role of cord blood transplantation, even though the content of NK in this source seems normal, the decreased IL-12 production by cord blood MNC, reducing IFN-γ stimulation, may contribute to reduce NK and LAK cytotoxicity; these data suggest one possible explanation for cord blood immaturity and their clinical implications such as decreased GvHD and GVL, which could be enhanced by IL-12 administration.[83]

Autologous setting. Considering the impressive results observed in the allogeneic setting using donor-buffy coat lymphocytes for treatment of relapse, CML seems an attractive field for testing the efficacy of adoptive immunotherapy in the autologous setting too; some experimental data support this hypothesis. The MNC of patients with CML contain a population of benign NK cells which can be expanded and activated by IL-2, generating a CTL population capable of killing both NK-sensitive and NK-resistant tumor targets.[84] Both number and functional activity of activated NK (ANK) in CML patients decrease with the progression of the disease.[85] *In vitro* data show that autologous ANK inhibit both committed and very early Philadelphia positive progenitors in a MHC-unrestricted manner.[86] In these experiments CML progenitor cell killing by autologous and allogeneic ANK (after T-cell depletion) was comparable. Finally the CML blast killing was not dependent of soluble factors because it was abrogated by a transwell membrane, but was mediated by cell-to-cell contact being significantly blocked by anti-integrin antibodies.[87]

In 1986 Lanier and Phillips described a subset of CD3$^-$ T cells co-expressing the CD56 antigen which is a typical NK marker (CIK).[88] More recently Schmidt-Wolf *et al.*[89] obtained large expansion of this subset in a 16-day liquid culture containing IFN-γ, IL-1, IL-2 and a monoclonal antibody against CD3 as the mitogenic stimulus. The same group tested the ability of this population to purge bone marrow in patients with CML; they found that while standard LAK cells were in most cases unable to lyse CML cells, CIK cells were able to lyse both autologous and allogeneic CML blasts, without affecting normal hemopoietic progenitors.[90] Recently it has been reported that CIK administration in SCID mice bearing human CML induced the disappearance of Ph'+ cells in the spleen of 12/14 animals.[91]

Another interesting potential application of autologous LAK is the treatment of EBV-related lymphomas arising in organ-transplanted patients; a preliminary description of four complete responses after treatment with autologous peripheral MNC incubated with IL-2 seems very promising.[92] Recently in thyroid cancer patients Katsumoto *et al.*[93] generated cytotoxic CD4$^+$ lymphocytes from TIL after non-specific *in vivo* stimulation with OK-432 (which induces severe local inflammation in the draining lymph nodes) and low-dose IL-2, obtaining large amounts of cytotoxic CD4$^+$ (Th1) cells, producing high levels of IFN-γ and TNF-β in the supernatants. These CE lysed a wide spectrum of tumor cell lines; anti-TCR antibodies did not inhibit their killing activity, which was in favor of a non-MHC restricted lysis, while antibodies anti-ICAM-1 completely inhibited the activity.

Tsurushima *et al.*[94] induced autologous CTLs directly from peripheral blood MNC by preparing a co-culture of minced tissue fragments of glioblastoma multiforme with a mixture of cytokines (IL-1, 2, 4, 6 and IFN-γ) for 2 weeks. At the end of culture the population contained mainly CD4$^+$ and CD8$^+$ lymphocytes able to kill 82 to 100% of the glioblastoma cells while normal LAK cells killed only 33%.

Finally, in follicular lymphomas freshly isolated TIL, normally lacking tumor-specific cytotoxicity, were stimulated with lymphoma cells, in the presence of IL-2 and CD40 ligand; these T-TIL were capable of proliferating in response to follicular lymphoma cells. moreover TIL could be further expanded in the presence of IL-4, IL-7 and IFN-γ.[95]

The potential role of new cytokines

Several cytokines affect CTL and NK response: first of all IL-2 which expands the precursor pool of allore-active CTL; IL-15 (producted by monocytes) mimics IL-2 action by inducing IFN-γ production, T-cell memory activation and CTL proliferation.[95] IL-12 shares certain functional properties with IL-2, but using a different, IL-2 independent pathway.[97] In addition IL-12 enhances the lytic activity of human peripheral blood MNC against a wide spectrum of tumors.[98,99] Recently it has been observed that the combination of IL-2 and IL-12 is capable of inducing lysis of blasts resistant to IL-2-activated effectors, even in the autologous setting.[100]

Therefore the association of IL-2 plus IL-12 could potentially become an important tool to increase the antitumor efficacy both *ex vivo*, by generating large amounts of CIK,[101] and *in vivo*, by systemic administration.

GM-CSF is a cytokine capable of inducing a pleiotropic immunostimulatory effect and also increases the immunogenicity of tumors; in a model for *ex vivo* expansion of LAK cells from leukapheresis in order to obtain contemporaneously a decontaminated harvest and a large amount of CE to reinfuse after myeloablative therapy, the association GM-CSF+IL-2 obtained a 5-fold expansion of the NK compartment while sparing the clonogenic potential of hemopoietic progenitors.[102]

Biodistribution and targeting of LAK and TIL

At present adoptive immunotherapy with LAK or IL-2 activated TIL has had limited success in patients with advanced cancer. Although a well-defined mechanism remains to be established, numerous *in vitro* findings and *in vivo* data suggest that the cancer-specific cytotoxicity of CE is obtained in multiple steps; a prerequisite, however, is optimal delivery of CE to the target tissues while minimizing systemic cytotoxicity. Two major areas currently requiring investigation are the survival and localization of adoptively transferred CE in the tumor-bearing host, and the detailed mechanism of tumor regression. The major goals in this area concern the optimal administration of systemic cytokines together with CE, and (finally) the ways to enhance localization and transcapillary migration of the infused cells.

Experimental evidence together with theoretical considerations based on CE functions indicate that the ability of adoptive immunotherapy to eradicate an established tumor is quantitatively determined by the initial tumor burden, growth pattern, and the magnitude of immunologic response generated by CE and other accessory cells at the site of the tumor. [illegible] Thus, to achieve tumor eradication and minimize systemic toxicity, the explanation of the mechanisms underlying lymphocyte biodistribution and the factors governing effector cell uptake in tumor sites is critical, but unfortunately data about CE biodistribution in humans are scarce.

Although a physiologically based kinetic modeling approach has been applied to the pharmacokinetics of drugs and antibodies, there has been no effort to extend this approach to cell biodistribution, probably because of its complexity.

One interesting attempt to apply this method to adoptive immunotherapy has, however, recently been published.[105] The importance of lymphocyte infiltration from surrounding normal tissues into tumor tissue was found to depend on lymphocyte migration rate, tumor size, and host organ.

It is likely that therapy with CE has not been as effective as originally promised, in part because of the very low CE concentration in the systemic circulation; this was mainly due to lung entrapment. Reducing this phenomenon by decreasing the attachment rate or adhesion site density in the lung by 50%, the tumor uptake could be increased by 40% for TIL to 60% for adherent NK cells.

Theoretical models indicate that intra-arterial administration has a dramatic advantage over intravenous delivery, with more than a 1,000-fold higher CE accumulation in the tumor site. Indeed experiments in murine models show that it is possible to eliminate liver metastasis by loco-regional administration of human IL-2 ANK or by systemic adoptive transfer.[106]

Finally the differences in biodistribution between different lymphocyte populations, mainly due to the different attachment rates in the tumor and the lung, should be carefully considered. ANK cells are more easily trapped than CTL in lung vessels due to their larger diameter and greater rigidity.[107] A greater accumulation of TIL was expected in the spleen as a result of their stronger adhesion at this site through the lymphocyte homing receptor.[108] Although this model has limitations related to the sensitivity of analysis of parameters such as adhesion-site density, lymphocyte attachment and arrest rate, it could be considered a useful basis for designing new experimental models to increase the concentration and recirculation of CE in tumor sites, reducing effector cell rigidity or blocking adhesion molecules.

The so-called antibody-dependent cellular cytotoxicity (ADCC) could be mediated by cells expressing Fcγ receptor II and Fcγ receptor III (e. g. NK cells and $CD3^+/CD16^+$ cells). This kind of cytotoxicity, even though exhibited by non MHC-restricted cells, cannot be considered aspecific and is also exhibited by monocytes.

LAK cells are extremely potent mediators of ADCC[109] and thus the use of LAK plus IL-2 in combination with monoclonal antibodies will probably become a powerful tool for treating some immunogenic tumors. This approach has been tested in patients with colorectal cancer [illegible] but could be also proposed for treating some immunogeneic hematologic malignancies such as follicular lymphoma or multiple myeloma.

Donor lymphocyte infusion for treatment of leukemia relapse and a a means for accelerating immunologic reconstitution in patients given transplantation of hematopoietic progenitors

Manipulation of the immune system after hematopoietic stem cell transplantation (HSCT) to reverse leukemia relapse or to reduce its incidence remains one of the most fascinating, even though difficult, challenges for successful cure of patients with hematologic malignancies. In fact, over the last 10-15 years, evidence has emerged from clinical transplantations to suggest that the anti-leukemia effect of allogeneic HSCT cannot merely be ascribed to the myeloablative therapy employed during the preparative regimen, donor lymphocytes playing a pivotal role in the eradication of malignant cells. Adoptive immunotherapy with donor lymphocyte infusion (DLI) in patients relapsing after HSCT has provided one of the most effective demonstrations of the importance of the graft-versus-leukemia effect in the cure of patients with hematologic malignancies.[111,112]

Even though DLI may sometimes be burdened by complications that endanger the patient's life, mainly myelosuppression and GvHD, in individuals with CML experiencing relapse in chronic phase after an allograft approximately 70% complete remissions can be obtained with this treatment.[112-116] Most of these remissions are sustained over time, this proving the capacity of DLI to eradicate clonogenic leukemia cells or control their re-growth. DLI has also been extensively employed to reverse relapse in patients with acute leukemia, non-Hodgkin's lymphoma and multiple myeloma. However, the response rate of patients with other hematologic malignancies, especially acute leukemia, is significantly lower.[115,116] In fact, only 20-30% of patients with AML achieve a hematologic remission after DLI and the value for patients with ALL is even lower. Patients with acute leukemia experiencing recurrence following an allograft have a higher probability of response with DLI if treated after having achieved a state of complete remission with chemotherapy, that is in a condition characterized by a limited tumor burden.[117]

The most important factor predicting response to DLI in patients with CML is the type of relapse. In fact, as already mentioned, patients suffering from cytogenetic relapse or hematologic relapse in chronic phase have a high probability of response to DLI, while patients with more advanced disease (accelerated phase or blast crisis) respond less frequently (20-25% of cases).[112-116] Relapse occurring in the first 1-2 years after allograft,[115] little or no acute and chronic GvHD after transplantation or removal of T-lymphocytes before HSCT[116] are also associated with a higher probability of benefitting from DLI. In patients with CML responding to DLI the median time to obtain hematologic remission has been reported to be about 6-8 weeks,[115] whereas a longer time (in the order of 11 months) is needed for molecular remission, this documenting that clearance of leukemia cells is a dynamic, progressive phenomenon.[118] The number of T-cells to be infused and the best schedule of DLI for optimal response without concurrent development of severe GvHD are still to be conclusively established since they depend on several variables, such as degree of HLA-compatibility between donor and recipient, original disorder, and type of relapse.[112] Some authors have claimed that infusion of no more than 1×10^7 donor-derived T-cells per kg of recipient body weight or CD8-depleted lymphocytes can induce a state of remission and substantially prevent GvHD occurrence.[119] However, recently, the Hammersmith Hospital group reported that the response in CML patients relapsing after HSCT and given graded increments of donor lymphocytes seems to be less sustained over time than that observed after infusion of a larger number (i.e. $>1\times10^8$/kg of recipient body weight) of T-cells (*Dazzi F, personal communication, 1999*). Support to the importance of the number of cells infused is also given by the results of Lokhorst *et al.*,[120] who observed that, in multiple myeloma, patients given more than 1×10^8 T-cells/kg had the highest probability of benefitting from DLI. In some of these patients, the response was complete with disappearance of myeloma proteins.

The two major complications occurring after DLI are myelosuppression and GvHD. Myelosuppression is experienced by approximately 50% of the patients treated with DLI for CML in hematologic relapse, while it occurs much less frequently in patients with cytogenetic recurrence,[112] this indicating that such a complication is observed in situations characterized by a predominance of host-type hematopoiesis. Therefore, myelosuppression can be explained by a direct effect of the transfused donor lymphocytes on hematopoietic cells of the recipient, similarly to that observed in transfusion-associated GvHD. The majority of patients experiencing myelosuppression after DLI recover a normal blood cell count spontaneously: nevertheless, myelosuppression may be fatal in approximately 10% of patients, with death being caused by infection or bleeding.[115,116] Infusion of a huge number of donor-derived peripheral blood hematopoietic progenitors, mobilized through hematopoietic growth factors such as granulocyte colony-stimulating factor (G-CSF), can alleviate the problem of pancytopenia in some selected cases, hastening the recovery of neutrophil and platelet counts.

Grade II-IV acute GvHD develops in almost half of patients given DLI,[115,116] the highest incidence being observed when the donor is an unrelated volunteer.[121] Incidence and severity of GvHD after DLI does not appear to correlate with GvHD after the original transplant and it may occur with a high incidence since donor lymphocyte therapy involves the infusion of large numbers of T-cells, whose immunocompetence

is not usually modulated by cyclosporin A and/or methotrexate. Even though GvHD occurring after DLI is well-correlated with disease response as proved by the observation that most patients obtaining a hematologic remission after this treatment developed acute and/or chronic GvHD, GvHD may not be sufficient to induce GVL. Moreover, some patients not experiencing GvHD after DLI achieve hematologic remission, this indicating the existence of a GVL effect separate from development of GvHD.[113,116,117,122]

GVL effect occurring after HSCT and DLI is considered to be mediated by HLA-unrestricted NK or LAK cells or by T-lymphocytes that recognize leukemia cells in an HLA-restricted fashion.[123,124] In particular, when patient and donor are HLA-identical, it is believed that recipient non-MHC-encoded minor histocompatibility antigens (mHAg) are recognized by donor CTL. While widely distributed mHAg account for the GVL effect associated to GvHD, tissue restricted or leukemia-specific antigens can elicit a specific GVL reaction[108,113] and it has been demonstrated that both $CD4^+$ and $CD8^+$ CTL recognizing mHAg in a classical MHC-restricted fashion can be generated *in vitro*.[124,125] In particular, mHAg-specific $CD8^+$ CTL can display strong lysis of mature leukemia cells, as well as suppress, together with $CD4^+$ mHAg-specific CTL, the growth of clonogenic leukemia precursor cells.[126,127] Production of cytokines (such as γ-interferon and tumour necrosis factor α) able to induce the apoptotic death of leukemia cells can also contribute to the GVL effect.[128,129] This said, it is not surprising that several efforts have been directed towards the identification of strategies capable of selecting and/or amplifying specific GVL response, not associated with development of GvHD. Since it has been documented in humans that CTL directed against allogeneic leukemic blasts can be detected in the peripheral blood of healthy donors[130] and that CTL specifically reactive towards recipient leukemic blasts can emerge and persist over time in children given allogeneic HSCT[131] a possible intriguing approach is that of generating and expanding clones or cell lines that are leukemia-reactive. The first elegant demonstration of the feasibility and efficacy of this sophisticated strategy has been recently reported by Falkenburg *et al.*,[132] who, through the infusion of donor-derived *in vitro* cultured CTL specifically recognizing leukemia progenitor cells, induced a complete hematologic and cytogenetic response in a patient with CML who had relapsed after an allograft and was resistant to DLI treatment.

A diverse, but equally elegant, approach proposed to abrogate the DLI-associated GvHD and its relevant morbidity and mortality is the infusion of thymidine kinase gene-transduced DLI followed by treatment of the recipient with ganciclovir if GvHD occurs.[133] In a study reported by Bonini *et al.*[133] this strategy proved to be able to control GvHD in 3 patients experiencing this complication after DLI; two of them, who had achieved a complete hematologic remission before ganciclovir administration, remained in full remission after disappearance of the transduced lymphocytes. If confirmed in a larger number of patients with a longer follow-up, genetic manipulation of donor lymphocytes, through the transfer of a suicide gene for specific and selective elimination of effector cells responsible for GvHD, could demonstrate the possibility of separating GvHD from GVL effect, thus sparing the anti-leukemia activity of DLI.

One of the most important, still unsolved problem of DLI is that concerning the much lower efficacy of GVL in patients with acute leukemia than in those with CML. An immediate explanation for this observation may be that the more rapid growth kinetics of blast cells, which occurs in patients with acute leukemia during the lag period between leukocyte infusion and GVL development, may hamper the immune-mediated effect played by donor lymphocytes in controlling disease progression. In fact, response to DLI occurs after weeks and hence the exponential expansion of leukemia cells *in vivo* may exceed the immune response.[112,113,124] The more encouraging results obtained when DLI is used as consolidation therapy for patients who have obtained a complete remission after chemotherapy provide support for this interpretation. However, other hypotheses, involving different intrinsic susceptibility of acute leukemia to adoptive immunotherapy must be considered. In particular, since patients with ALL have the lowest chance both of responding to DLI and of benefitting from the GVL effect after bone marrow transplantation,[134] a peculiar resistance of lymphoid leukemia to immunotherapy cannot be excluded.

As peptides differentially expressed within the hematopoietic system can trigger and act as a target of the GVL reaction,[112,124] it could be hypothesized that, for example, the presence of these antigens on myeloid blasts, but not on lymphoid leukemia cells accounts for the low response of ALL to donor lymphocytes. The reported demonstration of CTL response directed towards peptides derived from proteinase 3, which is expressed by myeloid cells (including blast cells),[135] is a typical example of the possible differential susceptibility to the immune-mediated anti-leukemia effect of different types of hematologic malignancies.

Several other possibilities exist to explain why acute leukemia (and in particular ALL) can escape the GVL effect. For example, leukemia cells may have defective expression of HLA-class I or II molecules on their surface such that they do not present antigens or, alternatively, the mechanisms of antigen processing and transport may be impaired.[112,129] Moreover, leukemia blasts may product cytokines (such as transforming growth factor β, IL-10) capable of suppressing T-cell activation, expansion and effector function or may express on their cell surface molecules, such as FAS ligand, able to mediate T cell apoptosis.[112,129] One of the most interesting fields of investigation for explain-

ing why in some patients a sustained anti-leukemia response *in vivo* fails to be induced is that of co-stimulatory molecules. As previously described, full activation of T-cells requires two distinct but synergistic signals.[136] In fact, in the absence of co-stimulatory signals, a T-cell encountering an antigen becomes unresponsive to the appropriate stimulation (anergic)[137] or undergoes programmed cell death (apoptosis).[138] Leukemia cells lacking these co-stimulatory molecules have a poor capacity of inducing a T-cell specific immune response and induction of CD80 and CD86, by signalling through the CD40 molecule, is able to restore T-cell co-stimulation via CD28 and to generate both allogeneic and autologous CTL, which could contribute to inducing or maintaining a state of hematologic remission.[139,140]

Some clinical strategies have been devised to improve the efficacy of adoptive immunotherapy in patients with acute leukemia. An approach for ameliorating the efficacy of DLI which has produced interesting results is that recently reported by Slavin *et al.*,[106] who documented that the success rate of this adoptive immune therapy may be increased in patients with both acute and chronic leukemia by activation of donor peripheral blood lymphocytes with IL-2 both *in vivo* and/or *in vitro*. In particular, a relevant proportion of patients who had not responded to DLI were induced into remission only after *in vivo* administration of IL-2 or *in vitro* activation of donor lymphocytes. If further confirmed, the results obtained make it possible to hypothesize that this strategy could be employed as first-line treatment of patients with acute leukemia relapsing after an allograft, since ALL and to a lesser extent AML patients do not greatly benefit from DLI alone. Another reasonable attempt for improving the response to DLI in patients with acute leukemia is to use this adoptive immunotherapy in individuals with minimal residual disease, as determined by cytogenetic investigations or sensitive molecular tools, that is in conditions characterized by a limited tumor burden, in which the GVL effect has demonstrated its greatest efficacy.

Unmanipulated DLI may also provide a means of compensatory T-cell repletion for the prevention of leukemia recurrence in patients given a T-cell depleted marrow transplantation from a relative. This approach has been recently proposed[141] and studies enrolling larger cohorts of patients are necessary to define whether this strategy can be useful to prevent the increased risk of relapse associated with the removal of donor T-cells. However, the main indication of adoptive infusion of donor immune cells to accelerate immune reconstruction after HSCT is transplants from HLA-disparate family donors. Infusion of a high number of T-cell depleted, peripheral blood hematopoietic progenitors from these donors has been demonstrated to be associated with a high chance (>95%) of donor hematopoietic engraftment.[142] The significant delay in immune reconstitution, due mainly to removal of mature T-cells from donor marrow and HLA disparity between donor and recipient, remains the major problem of HSCT from HLA-disparate donors. In fact, it is responsible for the dramatic incidence of leukemia relapse and life-threatening viral and fungal infections observed after this type of HSCT. A possible strategy to improve the process of immune recovery is to infuse donor T-lymphocytes selectively rendered non-reactive towards alloantigens of the recipient, but maintaining the capacity to generate an immune response against viruses, fungi and leukemia cells. In this regard, as previously mentioned, the manipulation of co-stimulatory molecules is an extremely promising field of investigation, since the absence of a second signal induces anergy rather than activation of T-lymphocytes. Drugs and monoclonal antibodies blocking co-stimulatory pathways have been demonstrated to be able to prevent T-cell activation in response to alloantigens and to induce a state of anergy.[143] In particular, it was recently documented that the combination of monoclonal antibodies blocking CD80/CD86 molecules and cyclosporin A was able to generate a state of selective *in vitro* unresponsiveness of T-cells towards allo-antigens, not reversed by adding IL-2.[144] Since the induction of this state of unresponsiveness was associated with the maintenance of *in vitro* capacity to respond toward virus antigens and leukemia cells,[145] the relevance of this approach is evident for strategies of donor T-cell add-back after T-cell depleted transplant of hematopoietic progenitors from HLA-partially matched donors aimed at accelerating the process of immune reconstitution.

A different, but equally promising, method of deletion of unwanted alloresponses is based on the elimination of alloreactive T-cells after specific activation through their killing[146] or fluorescence-activated cell sorting,[147] while sparing T-cells with other functions. In a human pre-clinical study, it was demonstrated that allospecific T-cell depletion by using an immunotoxin directed against the p55 chain of IL-2 receptor, was feasible and specific.[146] The spared T-cells were still able to proliferate against third-party cells, Candida and cytomegalovirus antigens,[148] as well as to kill both leukemia blasts and autologous EBV-B lymphoblastoid cell lines.[149] Moreover, *in vivo* studies in a murine animal model showed that this particular T-cell depletion was efficient, at least partially, in preventing both graft rejection and GvHD in a complete haplotype mismatched combination.[150]

Finally a brief mention should be made of the generation and infusion of T-cells with suppressive and regulatory activity. A particular subset of these cells called Tr1 has recently been described by Groux *et al.*,[151] who in an animal model demonstrated the ability of this population to prevent, through their activity on naive cells, the occurrence of ovo-albumin induced inflammatory bowel disease. Whether these

cells will have a role in promoting a true state of tolerance in transplant of hematopoietic progenitors or solid organs (in which the immune response to alloantigens is mainly sustained by memory cells) remains to be proved in specific pre-clinical and clinical studies currently underway.

Adoptive immunotherapy for the treatment of viral infections in immunocompromised patients

Prevention or treatment of viral infections in immune-compromised patients through the infusion of specific T-cell lines or clones is one of the most sophisticated examples of adoptive immunotherapy approaches.[152] In fact, it implies the elaboration of true cellular-engineering strategies able to generate, select and expand lymphocyte subsets, which display a specific function. The first study in humans to evaluate the efficacy of adoptively transferred T-cell clones for reconstitution of specific immunity was performed in recipients of allogeneic HSCT at risk of developing human cytomegalovirus (HCMV) infection and/or disease.[153] Even though pre-emptive therapy of HCMV infection based on monitoring of antigenemia[154] and prophylaxis of seropositive HSCT recipients using antiviral drugs (i.e. ganciclovir and foscarnet)[155] have significantly reduced the number of patients experiencing HCMV disease, this viral infection still represents a major life-threatening complication of stem cell allograft. The capacity to recover from a severe HCMV infection in transplanted patients is directly correlated with the ability of the host to generate virus-specific class I HLA-restricted $CD8^+$ cytotoxic cells and during the first 100 days after HSCT approximately 50% of patients are persistently deficient in $CD8^+$ cytotoxic T-lymphocytes specific for HCMV.[156,157] It is not surprising that, to evaluate the efficacy of adoptive immunotherapy in this viral infection, HCMV-specific $CD8^+$ T-cell clones of donor origin were generated and infused in HSCT recipients.[153,158] These cells, generated through a highly complex expansion strategy using irradiated donor-origin skin fibroblasts infected with a strain of HCMV, proved to be efficient in the prophylaxis against HCMV infections that can complicate allogeneic HSCT. Moreover, the cloning strategy allowed selection of T-cells which lacked significant alloreactive capacity and, thus, did not cause clinically relevant GvHD or toxicity. These clones, directed towards either pp65 or pp150 (two abundant viral tegument proteins presented for recognition by cytotoxic T-lymphocytes), restored HCMV-specific cytotoxicity, which persisted for several weeks.[158] In fact, through a PCR technique able to detect the Vα and Vβ T-cell receptor rearrangements specific for the donor clones, it was possible to prove the donor origin of these cells formally and to document the persistence of the adoptively transferred HCMV-specific T-cells for at least 12 weeks. Unfortunately, these clones persisted in the circulation at high levels only in patients experiencing an endogenous recovery of $CD4^+$ virus-specific cells.[158] By contrast, in patient lacking this spontaneous recovery of HCMV-specific $CD4^+$ lymphocyte, the donor-origin, adoptively transferred cytotoxic T-cell activity progressively declined and eventually disappeared. This observation emphasizes the importance of $CD4^+$ lymphocytes in promoting sustained restoration of antigen-specific immunity and suggests that the use of polyclonal T-cell lines containing both $CD4^+$ and $CD8^+$ cells could be preferable to the infusion of cytotoxic T-cell clones.

In this regard, the use of T-cell lines for prevention and/or treatment of Epstein-Barr virus-induced lymphoproliferative disorders (LPD) has represented a further, equally sophisticated, evolution of the approaches of adoptive immunotherapy for the restoration of virus-specific immunity. EBV-LPD have emerged as a significant complication for both HSCT and solid organ transplant recipients.[159-161] In the former cohort, the use of HLA-partially matched family and unrelated donors, as well as selective procedures of T-cell depletion sparing B-lymphocytes, are risk factors for the development of EBV-LPD.[160-162] In HSCT recipients these disorders are of donor origin and usually present in the first 4-6 months after transplantation, whereas in patients given a solid organ allograft they usually develop from the recipient B-lymphocytes months to years after transplantation.[160,161] High levels of EBV-DNA in blood and *in vitro* spontaneous growth of EBV-lymphoblastoid cell lines predict development of these lymphoproliferative disorders.[163] They often present as high-grade diffuse large cell B-cell lymphomas, which are oligoclonal or monoclonal and express the full array of EBV antigens including EBNA-1 through EBNA-6 and the latency membrane proteins LMP-1 and LMP-2.[161] The lymphomas which develop in immunocompromised hosts not only invade the hematopoietic system, but also the lung, nasopharynx and central nervous system. The therapeutic approaches proposed to date (i.e. discontinuation of immunosuppression, α-IFN, antiviral agents and cytotoxic chemotherapy) have been applied with varying, but overall unsatisfactory, results; moreover, graft rejection, GvHD and toxicity are frequent complications of these strategies, and mortality rate due to EBV-LPD remains high.[160,161]

Normal EBV seropositive individuals have a high frequency of circulating virus-specific cytotoxic T-lymphocytes precursors, which control outgrowth of EBV-infected B-cells. Since EBV-LPD in immunocompromised hosts appears to stem from a deficiency of virus-specific cytotoxic activity, it is reasonable to hypothesize that an adoptive immunotherapy approach with donor-derived T-lymphocytes could be able to prevent unchecked lymphoproliferation and eradicate established disease. In 1994, the Sloan

Kettering group first demonstrated that, through the infusion of unselected peripheral blood mononuclear cells from a donor, 5 patients given HSCT with post-transplant EBV-LPD obtained remission of the disease.[164] However, this treatment was associated with development of clinically relevant GvHD and 2 patients of inflammatory-mediated lung damage, leading to respiratory failure.

A further refinement of this approach was achieved by Rooney and colleagues, who generated EBV-specific T-cell lines from donor lymphocytes and infused them as prophylaxis against EBV-LPD in patients given T-cell depleted HSCT from HLA-disparate family or unrelated donors, and, thus, considered at high risk for this disease.[165] The infusion of these polyclonal T-cell lines proved to be safe and effective in the prevention of EBV-LPD. Moreover, these cytotoxic cells may also have a role in the treatment of established disease.[165] The most recent update of this experience confirms that the infusion of EBV-specific T-cell lines is highly effective for the prevention of EBV-LPD, since none of 39 patients given a T-cell depleted allograft and treated with this adoptive immunotherapy developed the disease, as compared to 7 out 61 transplanted patients not receiving the prophylactic treatment.[166] Gene marking studies have shown the persistence of these donor-derived EBV-specific cell cytotoxic lines in patient's peripheral blood for months after infusion and their re-appearance after periods of apparent non-identifiability during episodes of viral reactivation, this further stressing the importance of helper T-cell function in the persistence of transferred CD8+ cells.[167]

The profound immunosuppression necessary for graft survival carries a well-recognized predisposition to the development of viral complications, in particular EBV-LPD, also in recipients of solid organ transplantation.[159] An immunotherapy approach to EBV-LPD using autologous *in vitro* generated EBV-specific cytotoxic lines could be an appealing strategy in this cohort of patients. Support for this hypothesis is given by the recently described, although not unexpected, possibility of generating, from pre-transplantation blood samples of EBV-seropositive solid organ transplant recipients, virus-specific T-cell lines which are effective in controlling EBV replication post-transplantation.[168] However, generation and storage of cytotoxic lines for each patient undergoing solid organ transplantation requires enormous, unavailable levels of funding, laboratory facilities and workforce. A more rational strategy is to generate, expand and infuse autologous EBV-specific cytotoxic lines from the peripheral blood of organ transplant patients presenting increased EBV-DNA levels after transplantation, which, as previously mentioned, are a risk factor for EBV-LPD development. The feasibility of generating autologous EBV-specific cytotoxic lines from the peripheral blood of organ transplant patients receiving *in vivo* immunosuppression for prevention of graft rejection has been recently proved.[169] Moreover, these cytotoxic T-lymphocytes were demonstrated to be able to display EBV-specific killing *in vivo*, as proved by prompt viral DNA clearance, without augmenting the probability of graft rejection. A peculiar problem, fortunately not particularly common, is that of EBV-seronegative patients, who develop primary EBV infection after solid organ transplantation. In fact, in these patients, *in vitro* generation of virus specific T-cell lines able to control EBV-driven B-cell proliferation can be particularly complicated, time-consuming and sometimes unsuccessful.

Autologous EBV-specific cytotoxic lines with demonstrated anti-viral activity *in vitro* and *in vivo* may also have a role in the treatment of other EBV-associated primary malignancies: for example, 40-50% of patients with Hodgkin's disease tumor cells are EBV-antigen positive and may therefore be suitable targets for virus specific cytotoxic lymphocytes.[160,170] A recently reported study provides further support for this possibility, documenting that, although more complicated than in normal donors, generation of EBV-specific cytotoxic lines is feasible in a relevant proportion of patients with EBV-positive Hodgkin's disease.[171] These lines retained their potent antiviral effects *in vivo* and persisted for more than 13 weeks in patients with relapsed Hodgkin's disease.[171] Whether this approach of adoptive immunotherapy will become an adjunctive treatment option for patients failing to gain benefit from conventional chemotherapy remains to be proved in prospective clinical trials.

Finally, it should be mentioned that adoptive transfer of cytotoxic T-cell response could be of value also in the prevention or treatment of other viral infections that cause morbidity and mortality in immunocompromised patients. In this regard, pre-clinical studies are underway to establish systems for generating cytotoxic T-cell responses to adenovirus.[160,172]

Genetically engineered donor lymphocyte infusion for treatment of leukemia relapse and as a means of accelerating immunologic reconstitution in patients given transplantation of hematopoietic progenitors

Tumor recurrence is the major cause of treatment failure of autologous bone marrow transplantation.[173,174] Indeed, the rate of tumor relapse is lower when transplantation is performed between matched unrelated or mismatched family member donor and recipients. It is now established that the curative potential of allo-BMT is represented by the additional effect of high dose chemo-radiotherapy in addition to the presence of allogeneic T-lymphocytes that are responsible for the GVL.[175,176] However, the therapeutic impact of allogeneic BMT is limited by the inevitable occurrence of GvHD.[177] Severe GvHD can be circumvented by the *in vitro* removal of T-lympho-

cytes from the BMT.[175] However, recipients of depleted marrow have delayed immune recovery, and increased incidences of viral infections and tumor relapse.[178,179]

Recent studies have shown the clinical efficacy of the adoptive transfer of immune effectors specific for viral antigens[153,195,167] in patients who underwent BMT. In this context gene transfer of a marker gene provides a means of evaluating the survival, homing and efficacy of the infused cells.

In marrow-transplanted recipients, lymphoproliferative disorders associated with EBV, a human herpes virus that normally replicates in epithelial cells of the oropharyngeal tract, occurs in 5-30% of the treated patients. EBV-LPD are usually malignant B-cell lymphomas of donor origin, which may be either polyclonal or monoclonal. The latter have a rapidly progressive, fulminating and fatal course.[180,181] The transformed B cells express virus-encoded latent cycle nuclear antigens, latent membrane proteins, and a number of cell adhesion molecules. Most of these viral proteins are recognized as antigens by the immune system of a normal individual.[182] In the normal host, in fact, EBV-induced lymphoid proliferation is controlled by EBV-specific and MHC-restricted T-lymphocytes, MHC-unrestricted effectors and by antibodies directed toward specific viral antigens. Since a limited number of specific cytotoxic T-lymphocytes is required for controlling EBV-transformed B-lymphocytes in normal individuals, the administration of donor lymphocytes for the occurrence of EBV-LPD in recipients of T-cell depleted bone marrow transplantation could control this severe complication by providing the patient with donor immunity against EBV.[164,183] Successful regression of the disease, documented histologically and by full clinical remission, has been achieved by the infusion of unmanipulated donor leukocytes.[164] However, acute or mild chronic GvHD developed in all the patients who responded to the treatment.[164]

To prevent GvHD, Brenner's group has evaluated the use of EBV-specific CTL rather than unmanipulated T cells. Donor derived EBV-specific CTL have been generated *in vitro* by stimulation with irradiated donor-derived EBV-infected lymphoblastoid cell lines (LCL).[184] The polyclonal effector populations were predominantly $CD8^+$ with a varying number of CD4 and showed specific cytotoxic activity toward the EBV-infected target cells. In order to investigate the long-lasting survival of the injected cells, the anti-EBV effectors were marked with the neo-gene before administration.

Neo-marked cells were detected in circulation for at least 10 weeks after the injections.[165] Moreover, the infusions allowed the establishment of a population of CTL precursors that could be activated to proliferate by *in vivo* or *in vitro* challenge with the virus.[166] The authors showed that EBV specific CTL lines expressing the neo-marker could be derived from patient's peripheral blood lymphocytes (PBL) for up to 18 months, by *in vitro* restimulation with the autologous EBV-lines.[166]

These findings support a more widespread use of antigen-specific CTL in the treatment of infections and cancer. Their use may extend in the near future to other diseases which express well-known antigens that could serve as target of CTL therapy (e.g. Hodgkin's disease and nasopharyngeal carcinoma).

The adoptive transfer of *in vitro* stimulated effectors achieves clinical results without causing the appearance of GvHD. However, the application of this strategy to a large number of allo-BMT treated patients, especially in prophylaxis protocols has some limitations related to the *in vitro* manipulation necessary for the generation of specific effectors (e.g. availability of donor-EBV lines; *in vitro* stimulation and expansion of antigen-specific effectors). An alternative approach was proposed in 1994 by the S. Raffaele Hospital group.[185,186] Their protocol was aimed at maintaining the potential of the infusion of polyclonal cell lines while providing a specific means to control acute GvHD. To this aim they transduced donor lymphocytes by a retroviral vector containing a suicide gene for *in vivo* selective elimination of the infused lymphocytes.

It was previously shown that introduction of a gene encoding for a susceptibility factor, a so-called *suicide gene*, makes transduced cells sensitive to a drug not ordinarily toxic.[187,188] A series of retroviral vectors carrying a suicide gene for ganciclovir-mediated *in vivo* selective elimination of the infused lymphocytes was designed. The vectors carried either an HSV-thymidine-kinase-neo (Tk-neo) fusion gene, coding for a chimeric protein for both negative and positive selection, or the HSV-Tk gene alone.[189]

A crucial prerequisite for the application of this strategy in the clinical context is the transduction of all infused donor lymphocytes. For this purpose, the designed retroviral vectors also carried a gene encoding a modified (non-functional) cell surface marker not expressed on human lymphocytes. Positive immunoselection of the transduced cells[190] by the use of the cell surface marker resulted in virtually 100% gene-modified lymphocytes.

Based upon the preclinical data described above, a clinical protocol was developed[186] for the use of donor lymphocytes transduced by the SFCMM-2 retroviral vector for transfer and expression of two genes: the HSV-Tk gene that confers to the transduced PBL *in vivo* sensitivity to the drug ganciclovir, for *in vivo* specific elimination of cells potentially responsible for GvHD; and a modified (non-functional) form of the low affinity receptor for the nerve growth factor gene (ΔLNGFr), for *in vitro* selection of transduced cells and for *in vivo* follow-up of the infused donor lymphocytes.

Increasing doses (beginning at 1×10^6/kg) of donor PBL were infused into several patients affected by

hematologic malignancies who developed severe complications following a T-cell depleted BMT from their HLA-identical related donors. After the infusion, the transduced lymphocytes could be detected in the blood of patients by cytofluorimetric and PCR analyses. In particular one patient affected by an EBV-LPD, showed a progressive increase in the number (up to 13.4% of the total PBL) of infused marked lymphocytes that was accompanied by a complete clinical response. However, signs of acute GvHD, confirmed by skin biopsy, were observed approximately four weeks after the infusion of the transduced-donor lymphocytes. The intravenous (i.v.) administration of two doses of ganciclovir (10 mg/kg/day) quickly resulted in elimination of marked donor PBL, and near resolution of all clinical and biochemical signs of acute GvHD.[187]

As mentioned before, when comparable preparative regimens are employed, the rate of tumor recurrences after autologous BMT is significantly higher than the rate observed after allogeneic BMT. GvHD develops in 50-70% of patients undergoing allogeneic BMT. The effectors of such response are thought to be mature donor lymphocytes from the marrow graft that respond to the foreign major and/or minor histocompatibility antigens of the recipient and also recognize and destroy the tumor cells. In fact, patients who underwent mature T-cell-depleted allogeneic BMT have a lower rate of GvHD but also a higher rate of leukemia relapses.[178,179]

The infusion of donor lymphocytes, early after T-cell-depleted allogeneic BMT, increases the incidence of GvHD without improving the control of leukemia.[191] However, a delayed transfusion of donor lymphocytes, when graft tolerance is established, seems to be more effective in preventing and treating tumor relapses.

Indeed the delayed administration of donor lymphocytes has recently become a new tool for treating leukemic relapse after BMT. Patients affected by post-BMT recurrence of chronic myelogenous leukemia, acute leukemia, lymphoma, and multiple myeloma could achieve complete remission after the infusion of donor leukocytes without requiring cytoreductive chemotherapy or radiotherapy,[116,132,194] even though the response rate of patients with acute leukemia, non-Hodgkin's lymphoma and multiple myeloma is significantly lower than that of patients affected by chronic myelogenous leukemia. Although the delay in the administration of T-lymphocytes is expected to reduce the risk of GvHD, this risk is still present at higher doses of donor T-cells.[116] Therefore, as described above, a clinical protocol was developed, for the use of donor lymphocytes transduced by the SFCMM-2 retroviral vectors[186] for transfer and expression of the HSV-Tk gene, and the cell surface marker ΔLNGFr, for *in vitro* selection of 100% transduced cells and for *in vivo* follow-up of the infused donor lymphocytes.[190]

In a phase I-II study, eight patients affected by hematologic malignancies who developed severe complications following an allogeneic T-cell depleted BMT, received escalating doses of donor PBL transduced by the described retroviral vector.[133] After gene transfer, transduced cells were selected for the expression of the cell surface marker ΔLNGFr by the use of specific immunobeads and the proportion of transduced cells was assessed by cytofluorimetric analysis.[190] In this study, we made the following observations: 1) transduced cells survived long-term *in vivo* and were detectable by cytofluorimetric analysis and PCR in high proportions (up to 13.4% of circulating PBL) and long-term (up to 6 months); 2) three patients showed complete response, three patients had partial response, one progressed with no response, and one patient could not be evaluated; 3) three patients developed GvHD that required ganciclovir treatment; 4) ganciclovir-mediated elimination of transduced cells resulted in near resolution of all clinical and biochemical signs of acute GvHD. Data from this study[133] indicate that genetically modified cells maintain their *in vivo* potential to develop both anti-tumor and GvHD effect, and may represent a new potent tool for exploiting anti-tumor and anti-host immunity, while providing a specific means for eliminating acute GvHD, in the absence of any immunosuppressive drug.

A potential limitation of the clinical approaches described could be the development of a specific immune response against vector-encoded proteins, which might allow the selective elimination of the transduced cells by the host immune system. For some gene products, such as the hygromycin-thymidine kinase (Hy-Tk) fusion protein, a specific immune response, able to eliminate large numbers of transduced cells in less than 48 hours, has been described in HIV-patients.[195]

We observed that immune recognition and killing of cells transduced by retroviral vectors is a more general phenomenon related to the foreign nature of the proteins expressed by the injected cells. Indeed, cells expressing the widely used marker gene neo and the HSV-Tk gene are targets of a strong immune response, while the endogenous proteins (e.g. the cell surface marker ΔLNGFr) are not recognized, even if ectopically expressed in a context which is otherwise extremely immunogenic.[196] The relative immunogenicity detected for the three vector-encoded components (none by ΔLNGFr, low by HSV-Tk, high by neo) clearly outlined the modifications of this type of gene therapy. Since neo is the only component not strictly necessary for the strategy and can be efficaciously replaced by the surface marker for all *in vitro* handling and selection,[186] the immunogenicity of the new neo-less vectors should be reduced.

The clinical results obtained with gene modified donor lymphocytes, for the treatment of hematologic relapses and EBV-lymphoproliferative disorders,

suggest the potential use of this approach.[133] The transfer of a suicide gene, that allows selective and specific elimination of effector cells of GvHD may allow full advantage to be taken of the beneficial effect of allogeneic lymphocytes with the possibility of eliminating all unwanted effects of GvHD in the absence of toxic side effects. A large scale application of this strategy will increase the number of patients who could potentially benefit from allogeneic BMT by allowing the use of less compatible marrow donors.

With regard to the immune recovery associated with the genetically-engineered donor lymphocytes, our group has recently obtained *in vitro* data demonstrating that genetically-engineered donor T-cells maintain a normal TCR Vβ immune repertoire and retain antigen-specific lytic activity against an allogeneic target or an autologous EBV cell line at cytotoxic T-cell precursor frequencies comparable to unmodified lymphocytes. In the light of this *in vitro* evidence, and our previous clinical application,[133] a clinical trial, based on the prophylactic infusion of 1×10^7/kg HSV-Tk transduced T-cells six weeks after T-cell-depleted bone marrow transplantation, was developed. In the first five treated patients we documented the presence of various proportions of transduced cells in the peripheral blood. In particular, genetically-engineered donor lymphocytes were responsible for anti-viral immune reconstitution in one patient. CD3$^+$ lymphocytes began to appear in the circulation of this patient two weeks after the infusion of HSV-Tk T-cells. All the CD3$^+$ lymphocytes were genetically engineered as documented by the expression of the cell surface marker ΔLNGFr. These cells retained a polyclonal TCR repertoire and were probably responsible for the clearance of a persistent CMV antigenemia. Indeed, the CMV antigenemia dropped below levels which could be detected by PCR shortly after the appearance of circulating genetically-engineered CD3$^+$ T cells in the absence of any antiviral drug therapy.[198] These data, if confirmed in a larger number of patients with longer follow-up, suggest that in addition to the anti-tumor activity, the infusion of genetically-engineered donor lymphocytes may play a role in restoring immunity against opportunistic infections early after allogeneic BMT.

Dendritic cells as natural adjuvants in cancer immunotherapy

Among professional antigen presenting cells (APC), dendritic cells (DC) are specialized in capturing and processing antigens into peptide fragments that bind to major histocompatibility complex molecules. DC are the most potent stimulators of T-cell responses and they are unique in that they stimulate not only memory but also naive T-lymphocytes. Thus, DC appear critical (*nature adjuvants*) for the induction of

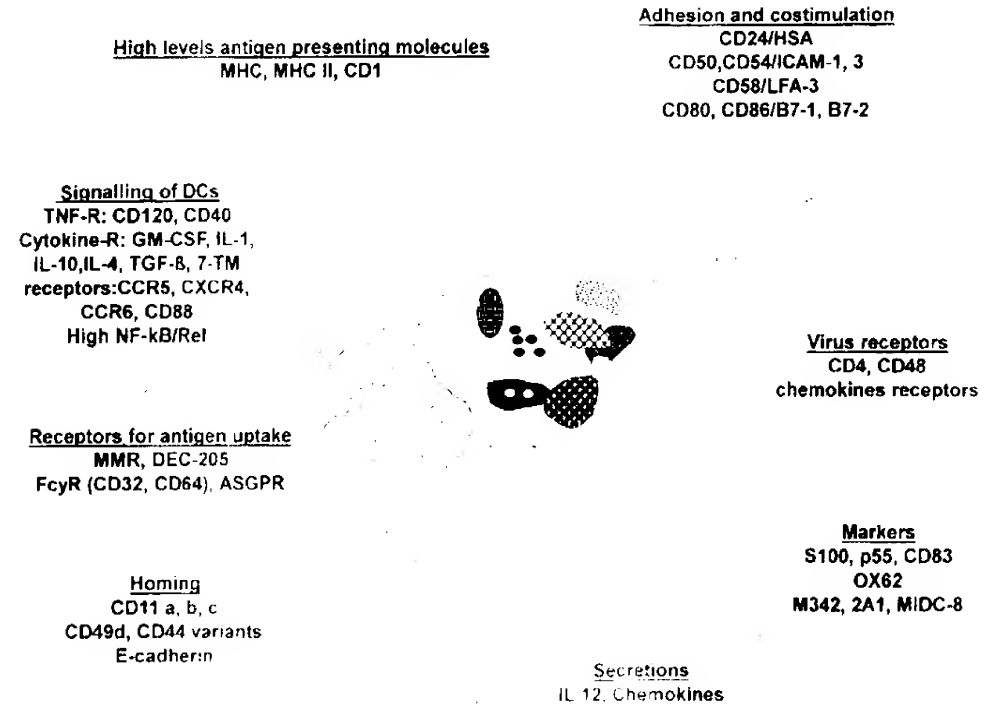


Figure 2. Phenotypic and functional characteristics of dendritic cells. *Modified from ref. #199 (Bancherau and Steinman, Nature, 1998).*

B-and T-cell-mediated immune responses. Recent evidence in experimental models supports the role of DC for immunization strategies aimed at stimulating specific anti-tumor immunity.

In this section we will briefly review:

1. the biological characterization of DC;
2. different strategies for *ex vivo* generation of DC;
3. methods for the efficient delivery of tumor associated antigens (TAA) to DC;
4. the use of DC for cellular immunotherapy.

Biological characterization of dendritic cells

DC are widely distributed in the body and are particulary abundant in tissues that interface the environment (i.e. Langerhans cells in the skin and mucous membranes) and in lymphoid organs (interdigitating DC) where they act as *sentinels* for incoming pathogens. Inflammatory signals such as TNF-α and IL-1β as well as bacteria, bacterial products (LPS) and viruses induce migration of antigen-loaded DC from the peripheral tissues to secondary lymphoid organs. During migration, DC mature and upregulate MHC, adhesion and co-stimulatory molecules, thus strongly augmenting their ability to prime T-cells.[199-204]

The functional activity of DC derives from a number of properties of these cells (Figure 2). Their dendritic shape, along with the high level of expression of certain adhesion molecules and integrins (LFA-3, ICAM-1, ICAM-3), increases the area of contact with the effector cells of the immune system.[205] DC strongly express the HLA class II molecules -RD, -DQ and -DP and co-stimulatory molecules (CD80, CD86 and CD40) which activate their ligands on T-cells (CD28, CTLA-4 and CD40L), thus providing the *second signal* strictly necessary to induce a proliferative response, rather than tolerance, upon antigen recognition.[199] In addition, DC produce a number of cytokines including IL-12 which promotes a cytotoxic immune response by inducing the differentiation of TH0 cells to IFN-γ and IL-2 producing TH1 cells.[206,207] It has recently been demonstrated that upon Ag recognition, T-helper cells activate DC via CD40-CD40L interaction and *activated* DC are then able to trigger a cytotoxic response from T-killer cells.[208-210]

However, DC are present in peripheral tissues in an *immature state* unable to prime T-cells. At this stage of differentiation, they can very efficiently take up soluble antigens, particles and micro-organisms by phagocytosis, macropinocytosis or by the macrophage mannose receptor, Fcγ and Fcε receptors,[211] but they lack all the accessory signals for T-cell activation. Antigen uptake induces DC to maturation by up-regulating MHC and co-stimulatory molecules as well as DC-associated Ag (e.g. CD83 and p55) whereas the capacity to capture and process Ag is lost. However, full activation of DC is dependent upon the contact with T-cells by the CD40-CD40L interaction which induces the production of IL-12. Thus, the key functions of DC (antigen uptake, T-cell stimulation) are strictly segregated to subsequent stages of differentiation (Figure 3). It is noteworthy that IL-10[212] and vascular endothelial growth factor (VEGF), secreted by cancer cells,[213] prevent the maturation of DC thus inhibiting the efficient priming of T-cells.

Different strategies for the generation of DC ex vivo

Circulating CD14+ monocytes represent the most readily available source of DC if incubated with appropriate cytokines such as GM-CSF, IL-4 and TNF-α.[214]

	DC IMMATURE	DC MATURE	DC ACTIVATED
Ag capture			
macropinocytosis	++	-	-
Mannose R	++	-	-
FcRs	++	-	-
T-cell stim.			
ICAM-1	-	+	++
MHC	±	+	++
B7-1,B7-2,CD40	±	+	++
IL-12	-	-	++

Figure 3. Functional properties of dendritic cells at different stages of differentiation. Pathogens or inflammatory cytokines induce the maturation of dendritic cells which become activated upon interaction with T-cells via CD40-CD40L.

[215] Moreover, DC precursors have been isolated within the CD34⁺ cell fraction in bone marrow, cord blood and steady state or mobilized peripheral blood.[216,221] Also in this case the differentiation of CD34⁺ cells into fully functional DC is strictly dependent upon stimulation with certain cytokines such as GM-CSF, TNF-α, SCF, FLT3-L and IL-4. An extensive review of the different types of human DC and their *ex vivo* generation is beyond the scope of this chapter. However, in view of the clinical use of DC a few critical points should be stressed. GM-CSF and IL-4 induce the differentiation of non-proliferating CD14⁺ monocytes to immature DC with a low level of expression of CD83 and p55 Ag and are largely incapable of priming naive T-cells. These immature DC are not fully differentiated and revert to an adherent state if the cytokines are removed from the culture medium.[222,223] The addition of inflammatory cytokines such as TNF-α, IL-1β or PGE2 for 1-2 days to the medium containing GM-CSF and IL-4 promotes the maturation of DC and increases the ability of stimulating T-cells. A potential bias toward the clinical use of this culture system is the requirement of fetal calf serum (FCS), a xenogenic protein that is contraindicated for human use. An innovative culture system for the generation of mature and functional DC from circulating monocytes that uses FCS-free conditions has recently been described.[222,223] In this system, adherent peripheral blood (PB) cells are cultured for 6-7 days with GM-CSF and IL-4 in the presence of FCS, which is then washed out, and subsequently exposed to macrophage-conditioned medium (Mo-CM) and 1-5% autologous plasma for 1-3 days. Mo-CM is very efficient in inducing the terminal maturation of DC and is prepared by growing T-cell-depleted PB cells on immunoglobulin (Ig)-coated Petri dishes for 24 hours.

Taken together, these findings lead to the conclusion that immature DC generated from CD14⁺ cells in the presence of GM-CSF and IL-4 are well equipped for capturing and processing soluble TAA. However, they do require a further maturation stimulus (Mo-CM, TNF-α) to exert their stimulatory effect on T-cells. Immature DC are the ideal targets for genetic manipulation using viral or bacterial vectors which infect non-replicating cells (see below). In this case, the modified pathogens can induce by themselves the full maturation of DC. In alternative, mature DC could be used in vaccination protocols involving TA peptides as DC also prime T-cells to foreign Ag that bind directly to MHC molecules without prior processing.[224]

As reported above, CD34⁺ cells can be induced to differentiate into fully functional DC which resemble cutaneous Langherans cells.[218] The issue of the large scale production of DC from CD34⁺ precursors has been discussed in detail elsewhere.[5] However, very recently the phenotypic and functional characteristics of DC derived from CD34⁺ cells mobilized into PB or from BM progenitors have been formally compared.[225] The published results indicate that G-CSF mobilizes DC precursors (CFU-DC) with an increased frequency and a higher proliferative capacity than their BM counterparts. This finding translates into a higher number of mature DC generated in liquid culture. Despite pre-treatment with G-CSF, these cells maintain the same functional capacity of stimulating allogeneic T-cells as BM-derived DC. CD34⁺ cell-derived DC are also capable of processing and presenting soluble Ag to autologous T-cells for both primary and secondary immune responses. The potential clinical usefulness of autologous serum in place of FCS[220] was also confirmed in the same study. Of note, IL-4 was shown to be capable of modulating DC differentiation from bipotent CD34⁺ cells during the later stages of the culture as previously demonstrated for monocyte-derived DC.[226] Thus, mobilized CD34⁺ cells may represent the optimal source for the generation of DC for cancer immunotherapy rather than BM precursors. Very recent data indicate the mobilization of large

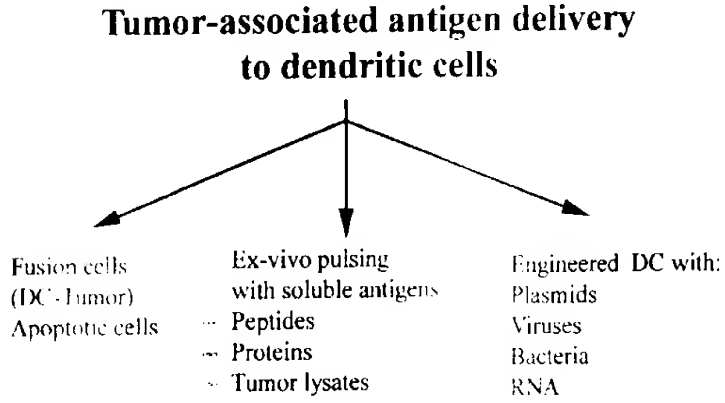


Figure 4.

numbers of DC precursors by GM-CSF[227] and FLT-3L.[228] However, it remains to be established whether circulating CD34⁺ elements are an equivalent source of DC to CD14⁺ monocytes. In this view, it has recently been demonstrated[229] that CD34⁺ cell-derived DC are more efficient than monocyte-derived DC, from the same patients, in stimulating a specific CTL response to Melan-A/Mart-1 peptides.

Delivery of TAA to DC

Several methods for the efficient delivery of TAA to DC have been described so far (Figure 4). Their rationale is based on the finding that tumor cells are often poorly immunogenic due to the lack of T-cell recognition, activation and co-stimulation typical of professional APC. To this end, Gong *et al.*[230] fused murine DC with the carcinoma cell line MC38 to provide tumor cells with the functional characteristics of DC. The fusion cells showed all the phenotypic features of DC and were shown to be capable of preventing tumor growth when the mice were challenged with the cell line. Moreover, treatment with fusion cells induced the rejection of pulmonary metastates.

Several TA peptides which are presented to T-cells in association with HLA class I molecules have been recently identified and proved to be useful in stimulating an autologous CTL response *in vitro* and *in vivo*. However, pulsing DC with peptides may not be optimal for clinical application because of the strict MHC restriction of the immune response and their limited stability. In addition, pulsing with peptides may not induce a T-cell response directed toward tumor cells expressing the relevant Ag. Although DC can be loaded with a cocktail of peptides from different Ag derived from the same type of cancer (see below), this vaccination approach is likely to limit patient selection on the basis of HLA phenotype. An attractive alternative is the use of unfractionated tumor-derived proteins, when available (see below), apoptotic cells[231] or tumor lysates. In the last case the obvious disadvantage is the possibility of inducing immune responses against self-Ag expressed in tissues other than tumor cells.

A further possibility is the transduction of DC with expression vectors encoding for TAA genes (Figure 4). DC can be engineered by different means which differ in their capacity of targeting quiescent cells, stable integration in the genome, infection efficiency and stimulation of anti-tumor immunity (Figure 5). Retrovirally-transduced DC constitutively express the relevant sequence and are potent stimulators of a specific T-cell response.[232] However, retroviral vectors have a relatively low efficiency of transduction, they can only infect actively replicating cells and carry the theoretical risk of oncogenic transformation of target cells. Conversely, adenoviruses infect both quiescent and proliferating cells and do not integrate into DNA.[233] Moreover, supernatants with a high titer of the virus can be easily obtained. Recently, DC have been transduced with an adenovirus combined with cationic liposomes showing an infection efficiency close to 100%.[234] The major limitation to the clinical use of adenoviruses is their high immunogenicity which induces the production of neutralizing antibodies and the rapid development of CTL directed at infected cells.

Vaccinia virus vectors are not oncogenic, do not integrate into genome and can be manipulated to

Gene delivery systems

	INFECTION REPLICATING CELLS		TARGET CELLS		DNA INTEGRATION (STABLE INTEGRATION)		INFECTION EFFICIENCY (%)	ANTI-TUMOR IMMUNITY (CTLs)
	YES	NO	CD34	DC	YES	NO		
PLASMID	+	+	+	+	-	+	1-10	+
VIRUS								
- RETROVIRUS	+	-	+	-	+	-	10-50	+
- ADENOVIRUS	+	+	+	+	-	+	80-100 *	+
- VACCINIA	+	+	+	+	-	+	50 °	+
BACTERIA	+	+	+	+	-	+	NA	NA
RNA	+	+	+	+	-	+	NA	+

* *Combined with cationic liposomes*
° *Low viability*

Figure 5.

carry large fragments of heterologous DNA.[235] However, these viruses are toxic for target cells and the viability of DC is approximately 50%. Nonetheless, antigen-specific inhibition of tumor growth has been observed in murine models using vaccinia vectors encoding for CEA and Mucin-1.[236,237] Two phase I clinical trials have been conducted to assess the safety of vaccinia virus vectors engineered to express HPV and CEA genes and to asses their capacity of stimulating an immune response.[238,239] More recently, maturation of DC with neo-biosynthesis, translocation and stabilization of MHC molecules on the cell surface and efficient induction of both CD4 and CD8 T-cell activation has been induced by infection with bacterial vectors.[240] As a result, a model Ag (ovalbumin) expressed on the surface of recombinant *Streptococco Gordonii*, is processed and presented on MHC class I molecules 10^6 times more efficiently than soluble OVA protein. Therefore, bacterial vectors are potentially useful means of delivering exogenous Ag to DC for stimulating a tumor-specific CTL response. A different approach has been taken by Boczkowsky *et al.*[241] who transfected DC with the total RNA extracted from tumor cells and combined it with cationic lipid to enhance the infection efficiency. Similarly to the use of tumor lysates, this strategy can be applied in those situations in which a tumor-specific antigenic marker is lacking; the major concern is the increased risk of autoimmune reactivity.

DC for cellular immunotherapy

The central role of DC in stimulating a tumor-specific immune response is well established *in vitro* and *in vivo* in animal models.[232,241-246] Whereas murine DC pulsed with TA-proteins or peptides or transduced with TAA genes have induced both the rejection of challenge tumor cells and the regression of established cancers, it remains to be determined which of the several strategies proposed for cellular immunotherapy is the most efficient. It may well be that different tumors require different approaches.

In humans, initial studies were performed in patients with melanoma using DC pulsed with MAGE peptide.[247,248] The infusion of loaded DC induced the migration of MAGE-specific CTL to the site of injection and increased the frequency of circulating tumor-specific CTL. More recently, Nestle *et al.*[249] have treated advance stage melanoma patients with intranodal injection of peptides or tumor lysates-pulsed DC according to the HLA profile of the patient. The authors reported the stimulation of a peptide specific T-cell response in all cases. Moreover, in 5/16 patients an objective clinical response was observed. In this study, DC were generated *ex vivo* from monocyte precursors in the presence of IL-4 and GM-CSF and directly injected into an inguinal lymph node to reach T-cell rich areas.

Tumor specific peptides (fragments of prostate specific antigen PSA) have also been used to pulse autologous DC in prostate cancer patients refractory to hormone-therapy.[250] Seven out of 51 patients showed a partial response while none of the patients in the control group, injected with peptides alone, showed any clinical benefit. In B-cell malignancies, the patient-specific idiotype (Id) gene sequence and its protein product represent the optimal targets for vaccination strategies as previously shown in murine models[251,252] and humans.[253] Hsu *et al.*[254] have reported on the treatment of 4 patients with low-grade non-Hodgkin's lymphoma (NHL), resistant to conventional chemotherapy or who had relapsed, with DC pulsed with the Id as soluble antigen. A tumor-specific T-cell-response was observed in all cases coupled, in one case, with the regression of tumor burden. At the time of writing, 16 patients have been treated and a tumor-specific cellular response has been found in 8 individuals (*R. Levy, personal communication*). The same strategy of targeting the Id has been proposed by the same group for inducing a T-cell immune response in multiple myeloma patients.[255]

In contrast to the strategy used by Nestle *et al.*[249] in this preliminary trial DC were freshly isolated from the PB by subsequent enrichment steps and were reinfused intravenously. Although a much larger number of DC were injected in NHL patients compared to melanoma patients ($3\text{-}20\times10^6$ DC vs 1×10^6), this approach raises concerns about both the efficacy of uncultured PB DC of efficiently stimulating T-cells and the capacity of Id-loaded APC to reach secondary lymphoid organs to prime T-cells, escaping the entrapment of the pulmonary apparatus.

Future directions

The few clinical data available so far have barely provided the *proof of principle* that autologous DC generated *ex vivo* and reinfused into cancer patients are effective in stimulating an anti-tumor immune response. This is the result of the complexity of the interplay between different cellular populations involved in tumor immunity. In addition, cellular immunotherapy with DC has yet to be standardized. As mentioned above, crucial issues such as 1) the choice of the most suitable TAA to stimulate an immune response; 2) the use of soluble proteins/peptides or DC engineered with expression vectors; 3) the optimal source for the generation of DC and the number of APC needed to promote a clinical effect; and 4) the most effective route of administration of DC, are points which still need to be solved. At this stage, relying for the most part on animal studies, we can only conclude that DC based immunotherapy holds promises of exerting a potent anti-tumor effect in humans.

Oral vaccination by in vivo targeting of DC

A simple approach to targeting APC *in vivo* is to use attenuated bacterial vectors, such as those commonly developed to control infectious diseases. They usually enter the host through the oral route and

selectively replicate within macrophages and DC. *Listeria monocytogenes* is a promising vaccine carrier that naturally infects APC, and may deliver immunogens to both MHC-I and II pathways of antigen processing and presentation.[256] Furthermore, this bacterium may constitute *per se* an excellent *danger signal* for the immune system, since it stimulates the innate immune response to produce cytokines (e.g. IL-12) and mediators (e.g. nitric oxide) that enhance antigen presentation. In addition, it promotes a TH1-type cellular response, which is mainly associated with the eradication of tumors and intracellular parasites. Most of these features are also shared by *Salmonella typhimurium*-based carriers.

The ideal vaccine carrier should maintain its immunogenicity intact, being attenuated enough to allow its use in humans. However, the safety profile of a vaccine destined for human use also requires the absolute stability of the mutant phenotype, which can only be guaranteed by the generation of chromosomal deletion mutants. Furthermore, the release of recombinant micro-organisms under uncontrolled conditions makes the lack of antibiotic resistance markers essential. Mutation of genes involved in bacterial spread and survival are the best targets for attenuation.

The recent progress in *Listeria* and *Salmonella* genetic manipulation and the availability of suitable *in vitro* and *in vivo* models, make these micro-organisms very attractive vaccine delivery systems.

For example, attenuated *Listeria monocytogenes* carrier strains expressing the β-galactosidase (β-gal) model antigen can prevent outgrowth of an experimental tumor in BALB/c mice by inducing a specific immune response against the β-gal TAA.[257] Similarly, a live attenuated AroA- auxotrophic mutant of *Salmonella typhimurium* (SL7207) has been used as a carrier for the pCMVββ vector that contains the β-gal gene under the control of the immediate early promoter of cytomegalovirus (CMV). After a primary immunization and three orally administered boosts at 15-day intervals, a *Salmonella*-based vaccine induced both cell-mediated and systemic humoral responses to β-gal. These experiments suggested that insertion of a plasmid containing an expression cassette into a *Salmonella*-carrier allowed DNA immunization and specific targeting of antigen expression to APC, *in vivo*, through oral immunization. To prove that the transgene was actually expressed by APC cells as a function of a eukaryotic promoter the green fluorescent protein (GFP) was placed under the control of either the eukaryotic CMV or a prokaryotic promoter and spleen cells from treated mice were analyzed by cytofluorometric analysis.

GFP was detectable in both macrophages and DC, but not in other splenocytes, of mice treated with *Salmonella* containing the CMV-plasmid, 28 days after the first vaccine administration, whereas it was undetectable in spleen cells of mice receiving the *Salmonella* containing the constitutive prokaryotic promoter which directs GFP synthesis only within the carrier.[258] GFP expression in DC highlights the possibility of loading DC without the need for *ex vivo* manipulations and opens up the possibility of administering a cancer vaccine orally. Oral vaccination is viewed as an easier and more acceptable strategy for patients especially in a phase in which they are disease-free.

Leukemic cells as antigen presenting cells

Tumors may escape immune detection and killing through a variety of mechanisms affecting the capacity of either presenting tumor antigens or fully activating T-cells.[258,260] In particular, tumor cells are likely to prevent a clinically evident cytotoxic T-cell response because of the absence of a specific antigenic tumor peptide, or because they lack HLA molecules, or co-stimulatory molecules on their surface. In this last case the patient's T-cells might become anergic and tolerate tumor cells. Alternatively, neoplastic antigens may induce a clonal deletion of thymocytes,[261] or tumor cells expressing Fas molecule may be responsible for an apoptotic T-cell deletion through Fas:Fasl interaction.[262] So far, different immunologic strategies aimed at overcoming these defects by inducing or improving the antigen presenting function of tumor cells have been demonstrated in experimental models,[263,264] and the hypothesis that leukemic cells may become efficient APC by changing their phenotype or by differentiating into DC-like cells has been tested. A first example was shown in B-cell neoplasms since it is well known that normal B-cells may present antigen to T-cells[265] and that cognate interactions between B- and T-cells may induce either a T-cell proliferation and an enhanced T-helper activity to cytotoxic T-cells,[266] or T-cell clonal unresponsiveness.[267] The triggering of the CD40 receptor on the surface of APC increases the expression of adhesion and co-stimulatory molecules both *in vitro* and *in vivo*.[269,269] Thus, the possibility of modifying the phenotype and the APC function of CD40$^+$-chronic lymphocytic leukemia B (CLL-B) cells through the CD40:CD40L interaction was demonstrated showing that this pathway induces the upregulation of CD80 and CD86 on CLL-B cells and the triggering of a T-cell proliferative response.[270,271] These results support the idea that induction of B7 molecules on CLL-B cells, either by T-cell-contact and growth factors,[270,272] or by gene transfer methods[273] may be a potential clinical vaccine-therapy capable of eliciting efficient anti-leukemic immune responses. Similar approaches may also apply to B non Hodgkin's lymphomas (B-NHL). Studies in experimental models indicated that CD40 stimulation may result in the inhibition of lymphoma cell growth *in vivo*,[274] and in the up-regulation of adhesion receptors and co-stimulatory molecules on lymphoma cells *in vitro*.[275] Interestingly, follicular B-NHL cells which express CD40 and low levels of B7-2 fail to present allo-antigen, but after activation via CD40 they express

higher levels of B7-1 and LFA-3 and alloreactive T-cells respond to tumor cells efficiently.[276] Finally, encouraging results have also been obtained in pre-B acute lymphoblastic leukemia[139] in which approximately 50% of the cases blast cells have been reported to express CD86 but not to induce tumor rejection, and B7-blasts determine an immunologic tolerance of the tumor. Nonetheless, this study showed that pre-activation of blast cells via CD40, or cross-linking CD28, or signaling through the common γ chain of the IL-2 receptor on T-cells can prevent T-cell tolerance. The authors hypothesize at least two possible mechanisms to explain the induction of lymphocyte unresponsiveness: first, they propose that at the time of initial transformation, clonogenic pre-B acute leukemia cells may not express CD86 thus inducing a T-cell anergy that could not be reversed by following expression of CD86 on a blast cell fraction; second, they suggest that marrow microenviroment may play a role in modulating T-cell immunity by secreting negative regulators, as previously shown in experimental models.[277,278] However, after co-stimulation by either B7 transfectants or professional APC, autologous antileukemic cytotoxic marrow T cells can be generated upon contact with CD40-stimulated pre-B acute leukemia cells.[140]

All these data on B-cell neoplasms strongly suggest that poor tumor immunogenicity may depend on both the quality and the quantity of accessory molecules required for T-cell stimulation. However, future therapeutic strategies aimed at stimulating the CD40 receptor, or at directly transducing B7 molecules on chronic or acute leukemia B-cells will facilitate the *ex vivo* expansion of specific anti-tumor cytototoxic T-cells. Normal myeloid CD34$^+$ progenitors include a small subset of APC[279,280] that are committed precursors of the macrophage/dendritic lineage.[281] In fact, both marrow and peripheral blood CD34$^+$ cells, and circulating monocytes can be utilized to obtain large numbers of dendritic cells *in vitro*. Due to the relevance of co-stimulatory molecules on tumor cells for the generation of anti-tumor immune responses, the hypothesis of whether even acute or chronic myelogenous leukemic cells might differentiate into dendritic cells *in vitro* and become immunogenic has been addressed by several groups. Alternatively, transduction of co-stimulatory molecules on leukemic myeloblasts has been attempted in experimental models to generate specific cytotoxic responses. Both these approaches require that TAA are expressed and exposed on HLA molecules, and it is likely that genetic alterations, such as chromosomic translocations, might result in the appearance of pathologic peptides, specific for each acute or chronic leukemia and potentially immunogenic. Chronic myelogenous leukemia may represent an optimal candidate for antitumor vaccine strategies since several reports have shown that the bcr-abl fusion protein can bind to defined HLA class I and class II molecules[282-286] and also that dendritic cells generated *in vitro* from CML patients still carry the t(9;22).[287,288] In this latter study, in fact, CML cells that were incubated with GM-CSF, IL-4 and TNF-α developed DC phenotypic and functional characteristics inducing autologous cytotoxic T-cells capable of directly lysing leukemic cells and of inhibiting CML colony growth *in vitro*. Further studies suggested that CML DC-stimulated anti-leukemic T-cell reactivity is due to an oligoclonal T-cell response and develops in an HLA-restricted manner [289] Dendritic cells can be generated even from CD34$^+$ CML marrow progenitors in the presence of GM-CSF, TNF-α and IL-4, and after 7-10 days of culture they are Ph$^-$, express high levels of HLA molecules and co-stimulatory receptors and induce a T-cell proliferation 10-30 fold higher than unprocessed marrow cells.[290] Nonetheless, it is likely that different culture systems may be required for efficient *in vitro* generation of DC when using CML-CD34$^+$ cells rather than normal progenitors, since the former show a lower DC clonogenic activity but both their expansion and their differentiation can be significantly improved by prolonging the duration of culture in the presence of specific growth factors.[291]

When a neoplastic event affects undifferentiated or more mature progenitors of the granulocytic and/or macrophage lineage an AML develops, and we can distinguish different subtypes of AML on the basis of morphologic and phenotypic characteristics. The identification of AML cells with some phenotypic affinities to DC, such as the expression of the CD1a marker,[292] or deriving from a monocytic/dendritic cell progenitor,[293] has been attempted in the past. Indeed in this latter study, cells from an AML, FAB M2 patient were shown to differentiate into terminal DC with potent alloantigen presenting capacity after *in vitro* culture with GM-CSF, TNF-α, SCF and IL-6. Similar results were achieved by culturing freshly isolated AML cells with GM-CSF, IL-4 and IL-13 for 7 days.[294] Alternatively, restoration of anti-tumor immune control can be attempted by identifying peptides, such as PR-1 derived from proteinase 3,[135] that could be capable of inducing HLA-restricted cytotoxic T-lymphocytes to lyse fresh leukemic cells, or by engineering leukemic cells to induce either the expression of co-stimulatory molecules or the production of cytokines. The role of B7-1 in developing protective immunity was initially tested in a mouse model in which the injection of a myeloid cell line transfected with the bcr/abl gene was rapidly lethal, while prolonged survival was observed only in mice that received the cell line co-transfected with the B7-1 gene.[295] Moreover, the same model was used to test the role of both B7-1 and B7-2, suggesting that B7-1 may be more effective than B7-2 in obtaining an efficient *in vivo* anti-leukemic response.[296] The potential advantage of B7-transduced blasts was confirmed by using primary AML cells instead of a cell line; a CD8$^+$ T-cell dependent and B7:CD28-mediated anti-leukemia activity was documented.[297] A recent study compared the *in vitro* immunogenic activity of human AML cells cultured with GM-CSF, IL-4 and

TNF-α, or transfected with CD80.[298] Both these approaches resulted in an enhanced T-cell response in a mismatched primary MLR, however, only B7-1 transduced AML cells stimulated a strong immune response of T-cells from an HLA identical bone marrow donor, and generated leukemia reactive CD4+ T-cell lines and clones. Interestingly, this model allowed the authors to observe CD80- AML-mediated T-cell responses that can be directed against the patient's minor histocompatibility antigens or tumor-specific antigens.

Although B7-1 and B7-2-engineered tumor cells could play a pivotal role in anti-leukemia immunotherapy strategies, there is evidence that transduction of other receptors[299] or cytokines[300-303] might, at least, co-operate with B7 molecules in the antigen presenting capacity of neoplastic cells.

Genetically modified cells as vaccine for the active immunotherapy of cancer

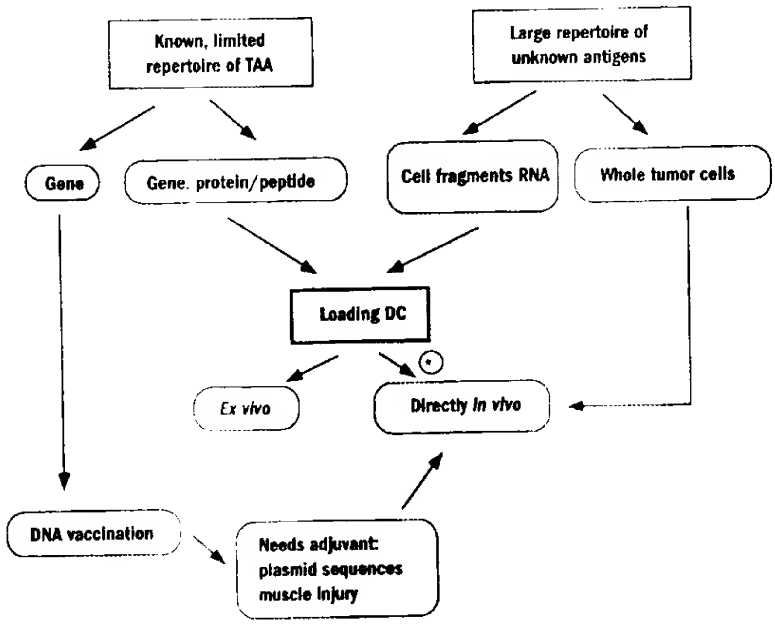
Non-specific approaches to cancer immunotherapy probably date back to the beginning of the 18th century and originated from the observation of sporadic, spontaneous remission of tumors in patients who suffered severe bacterial infection. This observation prompted Dr. William B. Coley to begin, in 1891, to treat patients with soft tissue sarcoma with a mixture of Gram positive and negative bacteria: Coley's toxins.

This empirical approach was enforced by Shear's discovery that endotoxins were active components responsible for tumor hemorrhagic necrosis. Furthermore, the finding that bacillus Calmette-Guérin (BCG) increased resistance to tumor transplants in mice led to clinical application of BCG which, together with *Streptococcus*-derived OK-432, is a strategy used to this day.

The anti-tumor effects obtained by treatment with BCG and derivatives are largely dependent on indiscriminate necrosis of tissues containing mycobacterium (the Koch phenomenon). The discovery of cytokines explained most of the phenomena induced by microbial products and cytokines were then used with the initial hope of copying the positive effects of such bacterial products while avoiding the negative ones.

More recently, the discovery of Th1 and Th2 distinct pathways of T-cell maturation helped to explain protective and non-protective BCG-induced cell-mediated immune reactions in tuberculosis, phenomena that have correlates with protection against cancer. In the presence of a Th1 deflected immune response, the effect of TNF-α is not that of large necrosis which is, rather, the characteristic of inflamed tissues of a Th2 type of response, in this case extremely sensitive to TNF-α.[304]

Cytokines deflecting the immune response to a Th1 or Th2 type of response may drive the type of immune response to cancer cells, escaping the simple definition of Th1 promoting and Th2 inhibiting anti-tumor immunity. Rather, a strong Th1 as well as a strong



Active immunization for cancer: strategies [illegible] whether tumor antigens have been [illegible] defined.

Figure 6.

Th2 response may induce tumor destruction and immune memory with the same efficacy although through different mechanisms (see below). Moreover, genetic background may influence the ability to mount a Th1 or Th2 response, as shown in murine models.

Microbial products have mainly local effects which may be reproduced and improved by local injection of recombinant cytokines. Experiments in non-tumor systems have shown that IL-2 offsets antigen recognition and overcomes tolerance. Thus cytokines could be used not only to stimulate tumor destruction but also to impair tolerance and activate effective and specific immune recognition of TAA.

Identification and cloning of the long elusive TAA, especially from human melanomas,[305] pointed tumor immunotherapy to a general systemic response and, thus, the use of cytokines shifted from that of being responsible for local tumor debulking to that of being an aid to triggering and boosting the immune response to TAA.

In addition to antigens triggering the T-cell-receptor (TCR) of T-lymphocytes, optimal T-cell response also requires co-stimulatory molecules, as detailed above.

Cytokines, co-stimulatory molecules and several cloned TAA are now available: how can we use them to provide an effective immunotherapeutic approach to cancer patients?

Two major strategies are envisaged (see Figure 6): one, already described, takes advantage of antigen availability in the forms of genes, proteins or peptides and of the standardized methods of obtaining DC from peripheral blood in large quantities to be loaded with the antigen and reinfused *in vivo*; the other strategy still considers the tumor cells representative of the entire antigenic repertoire of a certain neoplasia; such cells, genetically modified to produce cytokines and/or co-stimulatory genes, could be injected into patients as a cellular vaccine. In the latter case a pool of cell lines derived from different patients with the same type of tumor could increase the antigenic repertoire and avoid immunoselection that certain antigens may have encountered in some patients. Unmatched MHA are not a problem in terms of antigen presentation since injected cells are destroyed and represented by host APC. Moreover if different sets of alloantigens are selected from different pools, the risk of repeated alloimmunization during booster vaccination would probably be avoided. The background and prospectives of genetically modified tumor cell vaccines are presented below.

Cytokines at the tumor site

In initial studies recombinant cytokines were injected at the tumor site or cytokine genes were inserted into somatic cells to be injected at the tumor site. All these studies collectively established that most of the cytokines accumulated at the tumor site were able to induce tumor destruction and the reaction they induced was sometimes strong enough to eradicate a tumor antigenically unrelated to the cytokine-releasing cells. The obtained tumor debulking was often followed by a systemic tumor-specific immune memory. It should be underlined, however, that tumor debulking may occur through non-specific immune reactions or so fast as to prevent efficient T-cell priming, this being reminiscent of the dichotomy described for BCG: indiscriminate necrosis versus protective immunity.

Engineered tumor cell vaccines

Engineering of tumor cells with the gene of a particular cytokine is an efficient way of ensuring that this cytokine will be durably present at the tumor site. Repeated local injections would, of course, have the same effect. Bolus administration, however, does not provide a constant supply of cytokine. Its effects are much less evident than those achieved by the injection of engineered tumor cells[306] that can ensure the provision of antigen and continued local accumulation of the cytokine until a physiologic or a pharmacologic threshold is reached, and the biological activity of the cytokine can begin.

The immunogenicity that tumor cells can acquire upon cytokine-gene transduction may stem from recruitment by released cytokines, of particular repertoires of inflammatory cells, whose differing abilities to influence TAA presentation and secrete secondary cytokines may shape both immunogenicity and deflection of the ensuing immune memory towards a Th1 or Th2 type of response. A cytokine may be simultaneously involved in tumor rejection, leukocyte recruitment and activation of memory mechanisms.

Many experimental studies have been performed in mice over the last seven years and cytokine genes from IL-1 to IL-18 have been tested. Most of those studies described whether a certain cytokine gene, upon transduction, can inhibit tumor growth *in vivo*; some also described whether the cytokine induced protective immunization against challenge by parental cells whereas only a few studies described efficacy in a therapeutic setting. It is clear that the way cytokines modify tumor oncogenicity, immunogenicity and curative effect is not only dependent on the cytokine employed but also on the tumor model utilized. The immune mechanisms responsible for inhibition of tumor growth may not be the same as those required for immune memory or those necessary for eradication of an established tumor.

Translation of animal studies into a clinical setting faces a substantial difference, that is the fast growth of transplanted tumors and therefore the short time window in which immunization can be performed before the animal's death. In murine models, the so-called *established tumor* is a tumor that has been injected one to three days before the beginning of vaccination. This contrasts with phase I/II clinical studies in which enrolled patients have advanced disease. Clear

evidence of therapeutic effects is not expected in these patients, therefore tumors with antigens whose genes have been cloned and are recognized by CTL should be used to allow, at least, an immunologic follow-up that could prove the effect of vaccination. This confines the choice to those carrying the MAGE, GAGE and BAGE family genes and to melanomas, which also express antigens of the melanocyte lineage, such as tyrosinase, gp100 and MART-1/Melan-A.[305] The choice is further restricted by the difficulty of obtaining cells and cell lines from tumors that are not melanomas to be transduced and then employed for immunologic evaluation. Melanoma is thus the tumor most frequently chosen for vaccination studies.

Nevertheless, vaccination with cytokine-transduced, freshly isolated cells, which should retain the tumor-antigen repertoire, could be a way of generating tumor-specific T-lymphocyte lines and clones with which to identify antigens expressed by tumors other than melanomas.

In a few cases only, the antigens associated with the murine tumors employed in pre-clinical studies were characterized; the majority of studies designed to discover the immunologic mechanisms associated with tumor rejection utilized proteins not classifiable as tumor-associated antigens, such as β-galactosidase[244] and influenza nucleoprotein.[307] Most of these animal studies were carried out in the syngeneic system, that in humans corresponds to the autologous situation, in which a tumor cell line was both the cell vaccine and the tumor to be cured. Autologous application is actually difficult, since it requires tumor cell cultures from every patient for both gene transduction and immunologic follow-up. Each patient's cell vaccine should then be checked for safety, and a great variability in terms of cytokine production other than adhesion molecules and antigenic phenotypes may exist between cell vaccines. The use of allogeneic cell lines, on the other hand, has the advantage of employing vaccines well-characterized in terms of tumor antigen, MHC and adhesion molecules, as well as the constant amount of cytokine released; these parameters in combination may provide a standard reagent for clinical studies.

Both syngeneic and allogeneic tumor cells expressing a common TAA are processed by host APC such that TAA derived peptides are presented in association with host MHC in either case.[307] Nevertheless, in most clinical protocols the expression of the MHC class I allele, which presents TAA derived peptide(s), on the immunizing tumor cells is preferred. If cross-priming occurs efficiently, this should not be necessary, but it is still unclear whether vaccination with transduced tumor cells actually primes the host or boosts already present activated T-lymphocytes. This observation indicates that co-stimulatory molecules such as B7, in addition to cytokines may be transduced in cell vaccines in order to amplify the boosting effect, since is not clear whether B7 transduced cells prime the host directly.

Clinical vaccination protocols using IL-2 or IL-4 gene-transduced allogeneic melanoma cells have been performed at the Istituto Nazionale Tumori in Milan, Italy. An HLA-A2 melanoma cell line expressing Melan-A/MART-1, tyrosinase, gp100 and MAGE-3 has been transduced and irradiated before the treatment of advanced HLA-A2+ melanoma patients.[308] In the first protocol, patients were injected subcutaneously on days 1, 13, and 26 with IL-2 gene-transduced and irradiated melanoma cells at doses of 5 (3 patients) and 15 (4 patients) $\times 10^7$ cells. Mixed lymphocyte-tumor cultures (MLTC) and limiting dilution analyses were performed to compare pre- and post-vaccination PBL. While MLTC revealed an increased but MHC-unrestricted cytotoxicity, in two cases the frequencies of melanoma-specific CTL precursors were clearly augmented by vaccination. In one patient, HLA class II-restricted effectors were found to be involved in the recognition of autologous tumor. Which antigen(s) was involved in the recognition by PBL of vaccinated patients remains unclear. In 3 out of 5 cases studied, pre- and post-vaccination PBL could not recognize any melanoma peptide tested or known to be restricted by HLA-A2 allele.[308] Among other possible explanations, this might be due to a tumor associated antigenic repertoire that exceeds the limited number of antigens whose genes have been cloned so far.

This indicates that vaccination with cell lines is advantageous because the cell lines stimulate the host with the entire repertoire of known and unknown antigens. In the allogeneic system it is then easy to rotate the transduced cell line within the protocols and so maximize the chances that a relevant tumor antigen is present in the vaccine. Some antigens, in fact, may be negatively selected and lost in one patient-derived line, but not in others. In addition, selection of allogeneic cell lines displaying various MHC reduces the interference of repeated boosting with strong alloantigens. Indeed vaccination with a pool of three melanoma cell lines commenced before the cloning of known melanoma associated antigens, resulted in increased survival correlated with the level of antibody against the GM2 ganglioside, indicating possible involvement of a humoral response; correlation with the CTL response was not investigated.[309]

Going back to animal studies in which vaccination therapy with cytokine-transduced tumor cells was successful, it should be underlined that it was not clear which of the measured immune responses was responsible for the therapeutic effect since, generally, induction of cytotoxic T-lymphocytes was, *per se*, insufficient to produce a cure. In keeping with this statement, vaccination of 13 evaluable patients with MAGE-3.A1 peptide resulted in 5 clinical regressions, although no CTL precursors were found in the PBL of these responders.[310] Refined animal studies performed to identify which immune responses corre-

late with the therapeutic activity indicated that both T- and B-cells should be properly activated.[311,312]

These observations may suggest that while a patient could be immunized against a tumor, the immunity thus induced might be insufficient to fight the established tumor growing within its own stroma. The combination of poor immune function and large tumor burden makes patients with advanced disease dubious predictors of clinical response.

The general idea is that cytokine engineered tumor cells should be used as vaccines in minimal disease settings.[313] A new form of treatment would thus be available for combination with conventional management of patients after surgical removal of their tumor, patients with minimal residual disease, or patients expected to manifest tumor recurrence after a significant apparently disease-free interval. When compared with conventional forms of management, vaccination is a *soft*, non-invasive treatment, unlikely to cause particular distress or side-effects, and could be administered after resection of a primary tumor when recurrence is expected.

Use of mesenchymal cells for treatment of neoplastic and non-neoplastic disorders

In addition to hematopoietic stem cells which can differentiate to produce progenitors committed to terminal maturation,[314] human bone marrow also contains stem cells of non-hematopoietic tissues which are currently referred to as *mesenchymal stem cells* (MSC), because of their ability to differentiate into cells that can roughly be defined as mesenchymal, or as *marrow stromal cells* because they appear to arise from the complex array of supporting structures found in marrow.[315] Stromal cells of the marrow microenvironment include fibroblasts, endothelial cells, reticular cells, adipocytes, osteoblasts and macrophages, the last, although of hematopoietic origin, being considered functional components of the regulatory stroma.[316] The heterogeneous populations of mesenchymal cells and their associated biosynthetic products have the unique capacity to regulate hematopoiesis.[317]

Environmental components can modify the proliferative and differentiative behavior of hematopoietic cells by means of (i) cell-to-cell interactions, (ii) interactions of cells with extracellular matrix molecules, and (iii) interactions of cells with soluble growth regulatory molecules.[316] All these regulatory modalities participate in stromal cell-mediated regulation of hematopoiesis. In fact, marrow stromal cells provide the physical framework within which hematopoiesis occurs, play a role in directing the processes by synthesizing, sequestering or presenting growth-stimulatory and growth-inhibitory factors, and also produce numerous extracellular matrix proteins and express a broad repertoire of adhesion molecules that serve to mediate specific interactions with hematopoietic stem/progenitor cells of both myeloid and lymphoid origin.[318,319] Although growth factors play key roles in stem/progenitor cell proliferation and differentiation it seems improbable that hematopoiesis is regulated only by a random mix of growth factors and responsive cells. Rather, it is likely that regulatory molecules and localization phenomena within marrow stroma are required to sustain and regulate the function of the hematopoietic system.[320]

Although it is commonly accepted that stem cells are capable of homing to the marrow and docking at specific sites, the exact role of microenvironmental cells, adhesion molecules and extracellular matrix molecules in regulating the localization and spatial organization of hematopoietic stem cells in the marrow and driving myeloid and lymphoid regeneration following stem cell transplantation remains a matter of hypothesis.[321] Studies in animals demonstrated that stem and progenitor cells have different distributions across the femoral marrow cavity of mice, thus suggesting that marrow stroma is organized into functionally discrete environments, such as *primary microenvironmental* and *secondary microenvironmental* areas, allowing distinct differentiation patterns of hematopoietic stem cells.[322] The *stem cell niche* hypothesis, proposed by Schofield[323] suggested that certain microenvironmental cells of the marrow stroma could maintain the stem cells in a primitive, quiescent state. Another mechanism supporting the concept of specialized microenvironmental areas is stroma-mediated, compartimentalized growth factor production. Growth factor produced locally by stromal cells may bind to the extracellular matrix and be presented to immobilized target cells which recognize each growth factor through specific receptors.[320] This mechanism may provide the opportunity for localizing distinct growth factors at relatively high concentrations to discrete sites. As yet, relatively little is known of the nature of the factor(s) produced by different stromal cell types which modulate lineage development. However, a growing body of evidence suggests that marrow stroma is involved not only in regulating myeloid cell growth, but also in T- and B-cell lymphopoietic development.[324-327] Distinct adhesion molecules and cytokines are known to regulate stroma-dependent T- and B-lymphopoiesis,[328,329] suggesting that marrow stroma may function as a site of T- as well as B-cell lymphopoiesis.

The existence of self-renewing MSC is supported by several *in vitro* and *in vivo* data.[330] At the functional level, MSC residing within marrow microenvironment establish marrow stroma both *in vitro* and *in vivo* and have multilineage differentiation capacity, being capable of generating progenitors with restricted development potential which include fibroblast, osteoblast, adipocyte, chondrocyte and myoblast progenitors (Figure 7).[331-334] Putative stromal cell progenitors have been identified in human marrow by their ability to generate colonies of fibroblast-like cells originating from single clonogenic progenitors termed fibroblast colony-form

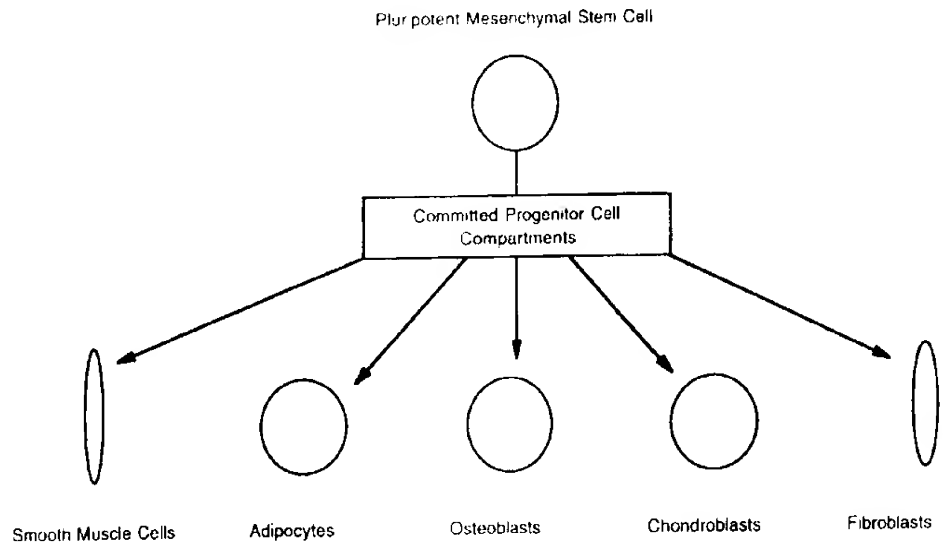


Figure 7.

ing units (CFU-F).[334] These progenitors, which belong to the osteogenic stromal lineage, play a central role in establishing the marrow microenvironment both *in vitro* and *in vivo*.[335-337] Under appropriate culture conditions and supplementation with specific stimuli, a proportion of marrow CFU-F can be induced to either adipogenesis[333] or osteoblastogenesis.[338] Studies involving ectopic transplantation of individual fibroblastic clones grown *in vitro* from mouse marrow beneath the renal capsule of syngeneic hosts demonstrated that approximately 15% produced a marrow organ containing the full spectrum of stromal cell types of hematopoietic microenvironment, thus suggesting that CFU-F have multilineage differentiation capacity and supporting the *stromal stem cell hypothesis*.[339] Based on these findings, CFU-F can be identified as multipotent stromal progenitors rather than lineage-restricted fibroblast progenitors.

CFU-F can be enriched from adult bone marrow by means of the STRO-1 monoclonal antibody that identifies essentially all assayable marrow CFU-F.[340] STRO-1$^+$ cells do not express the CD34 antigen and fail to generate hematopoietic progenitors, thus facilitating a clean separation between hematopoietic and stromal progenitors.[341] Flow-sorted STRO-1+ cells grown under long-term culture conditions generate adherent stromal layers consisting of fibroblasts, osteoblasts, smooth muscle cells and adipocytes.[340] These stromal layers are capable of supporting hematopoiesis in long-term cultures initiated with CD34$^+$ cells. In addition to STRO-1, other monoclonal antibodies, such as SH-2, have been described which specifically detect mesenchymal progenitors.[342]

In vivo data generated in animal models support the functional regulatory role of the marrow microenvironment. In the fetal sheep model of *in utero* stem cell transplantation, co-transplantation of stem cells with marrow stromal cells has been shown to improve levels of donor cell engraftment.[343] In the NOD/SCID mouse model of *in utero* stem cell transplantation, fetal stem cells have a nine times greater engraftment potential but this advantage is abrogated if the recipients are irradiated prior to transplant, indicating that the marrow microenvironment is important in driving myeloid and lymphoid engraftment.[344]

The importance of stromal cells in hematopoiesis has also been demonstrated by several studies in humans. Despite normal peripheral blood counts, levels of primitive and committed progenitors in the bone marrow of patients who have received allogeneic stem cell transplantation remain subnormal for many years.[345] Furthermore, cultured stromal cells from patients who have received allogeneic stem cell transplant (SCT) show significant impairment in their ability to support the growth of hematopoietic progenitors from normal marrow.[346] Decreased CFU-GM production and defective stroma production have been demonstrated following autologous SCT[347] as well as after induction chemotherapy.[348]

The role of marrow stroma in hematopoietic regulation and the peculiar functional characteristics of stromal cells raise the possibility that the delivery of *ex vivo* expanded marrow MSC into a hematopoietically-compromised marrow might promote hematopoiesis. Bone marrow stromal cells are a quiescent, non-cycling population with low cell turn-over, as demonstrated by the resistance to irradiation. Based on these characteristics, methods have been developed which allow for gene delivery into stromal cells.[349] Since stromal cells are metabolically active they also provide a

suitable means of secreting therapeutic proteins, including coagulation factors or adenosine deaminase.[350] Recent data showing that MSC suppress allogeneic T-cell responses *in vitro* suggest a role for stromal cells in modulating allogeneic transplant rejection and graft-versus-host disease.[351]

It must be emphasized that because of the limited knowledge of MSC biology, clinical applications of stromal cells, although exciting, essentially remain a matter of hypothesis to be carefully tested in the appropriate clinical setting. Essential prerequisites for clinical applications using culture-expanded mesenchymal cells as a supplement for hematopoietic SCT are (i) the possibility of isolating mesenchymal progenitors and manipulating their growth under defined *in vitro* culture conditions[352] and (ii) the demonstration of the possibility of efficiently introducing cultured stromal cells back into patients.

Studies in rodents and dogs have clearly demonstrated that if sufficient stromal cells are reinfused, they not only seed the bone marrow but also enhance hematopoietic recovery.[353-357] Although demonstrated in several mouse models, the *transplantability* of marrow stromal elements remains a controversial issue in humans.[358,359] The majority of data so far generated in recipients of HLA-identical marrow transplants has failed to demonstrate any contribution of donor cells to marrow stroma regeneration.[358] Although many factors may affect the *transplantability* of stromal elements, the low frequency of stromal progenitors in conventional marrow harvests may explain the failure of mesenchymal cell transplantation in humans.

Indeed, during the last decade, SCT methodology has changed substantially, particularly as a result of the increasing use of peripheral blood transplants. The existence of a circulating stromal progenitor has been demonstrated by using a NOD/SCID model and this is extremely relevant to stromal cell therapy.[360] By using the X-linked human androgen receptor (HUMARA) gene and fluorescent *in situ* hybridization analysis for the Y chromosome, the *transplantability* of stromal progenitors in a proportion of recipients of haploidentical HLA-mismatched T-cell-depleted allografts reinfused with a combination of bone marrow and mobilized peripheral blood cells has recently been demonstrated (*Carlo-Stella and Tabilio, unpublished observations, 1999*). Taken together, these findings allow the hypothesis that MSC are transplantable in man provided that an adequate, but as yet unidentified, number of CFU-F is reinfused. In addition, these data allow the planning of clinical studies using culture-expanded, gene-marked mesenchymal cells in order to investigate a number of issues, including (i) dose of marrow stromal progenitors necessary to achieve a transplant; (ii) duration of post-transplant marrow stromal cell function; (iii) role of stromal cells in myeloid, B- and T-lymphoid reconstitution following SCT.

Table 4. Potential clinical applications of mesenchymal stem cells.

- Replacement of chemotherapy-damaged stroma
- Enhancement of myeloid recovery following hematopoietic stem cell transplantation
- Enhancement of T- and B-cell reconstitution following allogeneic stem cell transplantation
- Compartimentalized growth factor/cytokine production
- Modulation of GvHD
- Delivery of exogenous gene products

A limited number of clinical trials using *ex vivo* generated MSC are currently underway. So far, the only published phase I clinical trial using MSC reported that the systemic infusion of autologous MSC appears to be well tolerated.[361] MSC can be explored as vehicles for both cell therapy and gene therapy (Table 4). MSC could be used to replace marrow microenvironment damaged by high-dose chemotherapy in order to either improve hematopoietic recovery from myeloablative chemotherapy or to treat late graft failures or delayed platelet engraftment. Based on their functional characteristics, MSC are attractive vehicles for gene therapy in that they are expected not to be lost through differentiation as rapidly as hematopoietic progenitors. Examples of diseases in which stromal cell-mediated gene therapy might be appropriate include factor VIII and factor IX deficiencies and the various lysosomal storage diseases. Interestingly, compared to skin fibroblasts or leukocytes, marrow-derived mesenchymal cells produce significantly higher levels of α-iduronidase, an enzyme involved in type II mucopolysaccharidoses (*Danesino and Carlo-Stella, unpublished data*). In addition, stromal cells might also be transduced with cDNA of various hematopoietic growth factors or cytokines. This approach might allow high levels of compartimentalized growth factor production and might be used (i) to stimulate hematopoiesis in patients with congenital or acquired hematopoietic defects, (ii) to improve B- and T-cell recovery following allogeneic SCT, (iii) to accelerate myeloid reconstitution in recipients of cord blood transplants.

In conclusion, MSC appear to be an attractive therapeutic tool capable of playing a role in a wide range of clinical applications in the context of both cell and gene therapy strategies. However, a number of fundamental questions about MSC still need to be resolved before they can be used for safe and effective cell and gene therapy.

Conclusions

Although most of the new therapeutic approaches of cell therapy are experimental and have not yet been validated by phase III clinical trials, they appear to

hold a high therapeutic potential. Separation of GVL from GvHD through generation and infusion of leukemia-specific T-cell clones or lines is one of the most intriguing and promising fields of investigations for the future. Likewise, strategies devised to improve immune reconstitution and restore specific anti-infectious functions through either induction of unresponsiveness to recipient alloantigens or removal of alloreactive donor T-cells might increase the applicability and success of hematopoietic stem cell transplantation. Cellular immunotherapy with DC must be standardized and several critical points, discussed in this review article must be properly addressed with specific clinical studies. Stimulation of leukemic cells via CD40 receptors and transduction of tumor cells with co-stimulatory molecules and/or cytokines may be useful in preventing tumor escape from immune surveillance. Tumor cells can be genetically modified to interact directly with dendritic cells *in vivo* or recombinant antigens can be delivered to dendritic cells using attenuated bacterial vectors by oral vaccination. MSC represent an attractive therapeutic tool capable of playing a role in a wide range of clinical applications in the context of both cell and gene therapy strategies.

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All authors gave substantial contributions to analysis and interpretation of literature data and drafting the article or revising it critically. CB was primarily responsible for the section on genetically engineered donor lymphocyte infusion for treatment of leukemia relapse, CCS for the section on mesenchymal cells, MPC for the sections on oral vaccination and engineered tumor vaccines; RML for the section on dendritic cells DR. FL was primarily responsible for sections on adoptive immunotherapy, AO for the sections on LAK and TIL and DR for the section on tumor escape from immune surveillance and on leukemic cells as APC. The authors are listed in alphabetical order.

Disclosures

Conflict of interest. This review article was prepared by request from Haematologica. The authors were a group of experts and representatives of two pharmaceutical companies, Amgen Italia SpA and Dompé Biotec SpA, both from Milan, Italy. This co-operation between a medical journal and pharmaceutical companies is based on the common aim of achieving optimal use of new therapeutic procedures in medical practice. In agreement with the Journal's Conflict of Interest policy, the reader is given the following information. The preparation of this manuscript was supported by educational grants from the two companies. Dompé Biotec SpA sells G-CSF and rHuEpo in Italy, and Amgen Italia SpA has a stake in Dompe Biotec SpA.

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